EVALUATION OF IN VIVO ANTICANCER ACTIVITY OF ETHANOLIC EXTRACT OF LEAF OF *Plumeria acutifolia* ON DALTONS ASCITES LYMPHOMA INDUCED CANCER IN MICE

Dissertation submitted to

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY,

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In partial fulfillment for the award of the degree of

MASTER OF PHARMACY

IN

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Submitted by

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Under the Guidance of

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Tamil Nadu.

APRIL-2017

CERTIFICATE

This is to certify that the work embodied in this dissertation entitled "**Evaluation of in vivo anticancer activity of ethanolic extract of leaf of *plumeria acutifolia* on Daltons ascites lymphoma induced cancer in mice"**, submitted to "The Tamilnadu Dr.M.G.R. Medical University", Chennai. in partial fulfillment to the requirement for the award of Degree of **Master of Pharmacy in Pharmacology**,is a bonafide work carried out by **Mr.N.YOGESH, Reg.No-261525216**, during the academic year 2016-2017, under my guidance and direct supervision in the department of pharmacology, J.K.K.Nataraja College of Pharmacy, Komarapalayam.

Internal Examiner **External Examiner**

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I further declare that, this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma associate ship and fellowship or any other similar title.

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CONTENTS

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1.INTRODUCTION

MEDICINAL PLANTS

Various plants have been used for medicinal purpose long before prehistoric period. Traditional systems of medicine continue to be widely practiced on many accounts. population rise inadequate supply of drugs prohibitive cost of treatments, side effects of several synthetic drugs and development of resistance to currently used drugs for infection disease have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments. Among ancient civilization india has been known to be rich repository of medicinal plants. About 8000 herbal remedies have been codified in AYUSH systems in india. ayurveda, unani, siddha and folk (tribal) medicines are the major systems of indigenous medicines. Among these systems, ayurveda and unani medicines are most developed and widely practised in india.according to WHO around 21000 plant species have the potential for being used as medicinal plants.The economic importance of medicinal plants is much more to countries provide two third of plants used in modern systems of medicine and health care system of rural population depend on indigenous systems of medicine. Treatment with medicinal plants is considered very safety as there is no or minimal side effects. there remedies are in sync with nature.which is biggest advantage. The golden fact is that use of herbal treatments is independent of any age groups and sexes.The most of the drugs thus formulated are of side effects or reactions.this is the reason why medicinal treatment growing in popularity across the globe. Medicinal plants are considered as a rich resources of ingredients which can be used in drug development either pharmacopoeial or synthetic drugs.

WHY PEOPLE WITH CANCER USE NATURAL MEDICINES.

People with cancer offen use touch therapies such as massage and aroma therapy,etc. Many people say these therapies help them to cope better with cancer and its treatment.Research is looking into whether some herbs or plant treatments used in ayurvedic medicine could help to prevent or treat cancer.But we still don't know much about some of the treatments that are part of ayurvedic medicine such as special diets and herbal remedies.

Although great advancements have been made in the treatment and control of cancer. progression significant deficiencies and room for improvement remain. plants have enormous potential to provide newer drugs and as such are a reservoir of natural chemicals that may provide chemo productive potential against cancer. A number of side effects sometimes occur during chemotherapy. Natural therapies such as the use of plant derived products in cancer treatment may reduce adverse side effects. Currently a few plant products are being used to treat cancer. However a myriad of many plant products exist that have shown very promising anti cancer properties in vitro but rare yet to be evaluated in humans.

Further study is required to determine the efficacy of these plants products in treating cancer in humans

TUMOR[1]:

Neoplasia literally means the process of "new growth," and a new growth is called a neoplasm. The term tumor was originally applied to the swelling caused by inflammation. Neoplasms also may induce swellings, but by long precedent, the non-neoplastic usage of tumor has passed into limbo; thus, the term is now equated with neoplasm. Oncology (Greek oncos = tumor) is the study of tumors or neoplasms. Cancer is the common term for all malignant tumors. Although the ancient origins of this term are somewhat uncertain, it probably derives from the Latin for crab, cancer-presumably because a cancer "adheres to any part that it seizes upon in an obstinate manner like the crab."

1. DEFINITIONS:

Solid tumors are defined as abnormal masses of tissue that usually do not contain cysts or liquid areas. Solid tumors may be benign (not cancerous), or malignant (cancerous). A number of malignant diseases are often also categorized as “solid tumors” such as breast cancer, cancer of the pancreas, lung, colon, etc.

Solid tumors can be split into three separate categories, depending on the type of cells from which they typically arise in the patient’s body, which include:

- **Sarcomas:** Cancers arising from connective or supporting tissues such as bone or muscle.
- **Carcinomas:** Cancers arising from the body's glandular cells and epithelial cells, which line the air passages and gastrointestinal tract.
- **Lymphomas:** Cancers of the lymphoid organs such as lymph nodes, spleen, and thymus, which produce and store infection-fighting cells. Lymphoma is cancer of the lymphatic system, which is part of the immune system.

Lymphoma is a form of cancer that affects the immune system - specifically, it is a cancer of immune cells called lymphocytes, a type of white blood cell. There are two broad types of lymphoma and many subtypes.

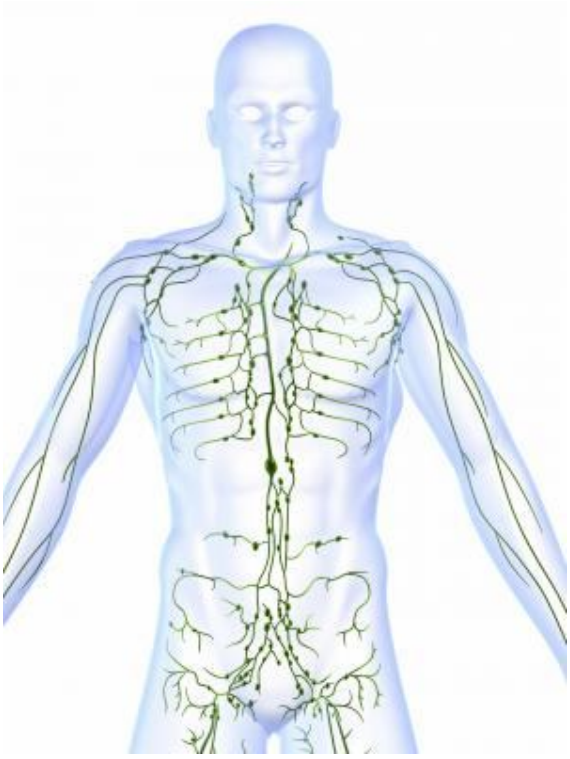
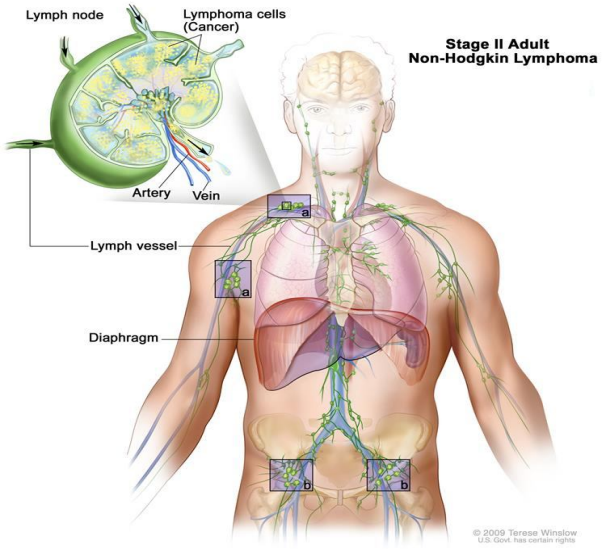


Figure.1 *The lymphatic system is a system of vessels that branch back from virtually all our tissues to drain excess fluids and present foreign material to the lymph nodes.*

The two types of lymphoma are described as: **Hodgkin's or non Hodgkin's**. Lymphoma can occur at any age but is the most common cancer in young people. It is often very treatable, and most people live for a long time after being diagnosed.

About 90% of lymphomas are the non-Hodgkin's type while about 10% are Hodgkin's.

Cancer is a group of over 100 diseases, all of which start with the growth of abnormal cells. Instead of dying in the normal cell life cycle, cancerous cells continue to divide into new abnormal cells, and grow out of control.



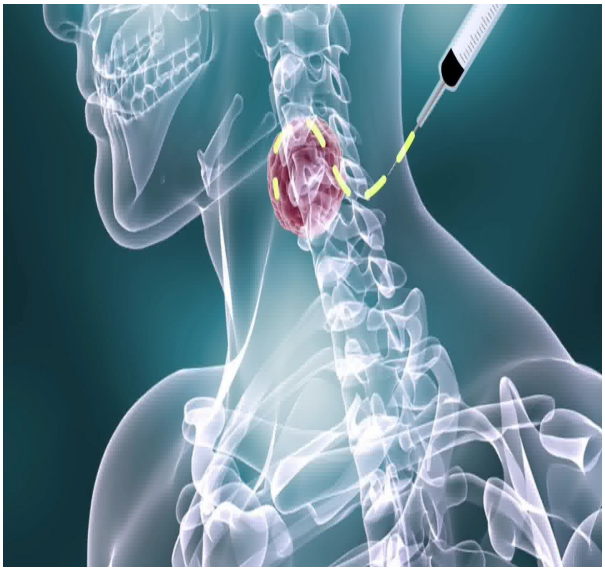


figure.2 lymphatic cancer

Lymphatic cancers are classified by the type of immune cells affected.

In non-Hodgkin's lymphoma, B-cells and T-cells are affected - both being types of lymphocyte white blood cell with special roles in immunity. In the US, B-cell lymphomas are much more common than T-cell ones.

In Hodgkin's lymphoma, the cancer cells are usually an abnormal type of B lymphocyte, named Reed-Sternberg cells. There are many subtypes of Hodgkin's lymphoma, typed by differences seen under the microscope - but a very high percentage of cases are classed as "classic" Hodgkin's.

Types of lymphoma

There are many different types of lymphoma depending on the type of lymphatic cells affected.

Hodgkin's lymphoma can occur at any age, affects more men than women and the majority will be completely cured.

Hodgkin's lymphoma is diagnosed when a special type of cell, the Reed-Sternberg cell, is seen under the microscope.

Non-Hodgkin's lymphoma accounts for all the other types of lymphoma. These can be high grade or low grade and the treatment and prognosis varies.

Factors for Non-Hodgkin's lymphoma

- **Age** - most non-Hodgkin lymphomas are in people 60 years of age and over
- **Sex** - there are different rates of different types of non-Hodgkin's lymphoma across the sexes
- **Ethnicity and location** - in the US, African-Americans and Asian-Americans are less prone than white Americans, and the disease is more common in developed nations of the world
- **Chemicals and radiation** - some chemicals used in agriculture have been linked, as has nuclear radiation exposure
- **Immune deficiency** - for example, caused by HIV infection or in organ transplantation

- **Autoimmune disease**, in which the immune system attacks the body's own cells
- **Infection** - certain viral and bacterial infections increase the risk. The Helicobacter Infection has been implicated, as has the Epstein Barr Virus (the virus that causes glandular fever)
- See the American Cancer Society's page for more detail on risk factors for non-Hodgkin's lymphoma.

Factors for Hodgkin's lymphoma

- **Infectious mononucleosis** - infection with Epstein-Barr virus
- **Age** - two specific groups are most affected: typically people in their 20s, and people over the age of 55 years
- **Sex** - slightly more common in men
- **Location** - most common in the US, Canada and northern Europe; least common in Asia
- **Family** - if a sibling has the condition, the risk is slightly higher, and very high if there is an identical twin
- **Affluence** - people from higher socioeconomic status are at greater risk
- **HIV infection**
- See the American Cancer Society's page for more detail on risk factors for Hodgkin's lymphoma.

Symptoms of lymphoma

The symptoms and signs of lymphoma are very similar to those of simple illnesses such as viral illnesses and the common cold, and this can cause problems with delayed diagnosis.

The difference is that the symptoms of lymphoma persist long after the usual run of a viral infection.

- Swelling in the legs or ankles
- Cramping and bloating of the abdomen
- Night sweats and fever
- Weight loss and loss of appetite

- Chills
- Unusual itching
- Fatigue
- Pain or altered sensation
- Loss of appetite
- Unusual tiredness/lack of energy
- Persistent coughing
- Breathlessness
- Enlarged tonsils
- Headache.

Treatments and prevention

A number of treatment options are used against lymphoma cancer these are

- Radiation therapy
- Biologic therapy
- Antibody therapy
- Stem-cell transplantation
- Splenectomy
- Steroid treatment
- Radioimmunotherapy
- Surgery.

1.2 ANTIOXIDANTS:

Free radical is a chemical compound which contains an unpaired electron spinning on the peripheral layer around the nucleus. The family of free radicals generated from the oxygen is called ROS which cause damage to other molecules by extracting electrons from them in order to attain stability. ROS are ions, atoms or molecules that have the ability to oxidize reduced molecules. ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals ($O_2^{\cdot}$) And hydroxyl radicals (OH), as well as non-free radicals (H_2O_2) and singlet oxygen[2]. In the body, free radicals are derived from two sources: endogenous sources, e.g. Nutrient metabolism, ageing process etc and exogenous sources e.g. Tobacco smoke, ionizing radiation, air pollution, organic solvents, pesticides[3].

When oxygen traps single electron, it becomes unstable and thus very reactive, since it generates harmful chain reactions against many biological molecules. The extreme toxicity of oxygen is related to its high capability of generating free radicals and in turn destroying many major biological molecules. They can attack on lipids and proteins and destroy membranes. ROS can damage DNA and lead to mutation and chromosomal damage. Oxidized cellular thiols abstract hydrogen atoms from unsaturated fatty acids to initiate the peroxidation of membrane lipids[4]. ROS can attack various substrates in body and contribute to development of chronic diseases. Exogenous chemicals and endogenous metabolic processes in human body produce free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules, resulting in cell death. Superoxide anion radicals increase under stress conditions such as heavy exercise, certain drugs, infection and various disease states. During normal metabolic processes, human body generates more than 2 Kg of O_2 per year[5].

Cells are equipped with different kinds of mechanisms to fight against ROS and to maintain the redox homeostasis of cell. For example, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) play important roles inscavenging the free radicals and preventing cell injury[6]. Molecules such as vitamin C and E inhibit lipid peroxidation in cell. When

the mechanism of antioxidant protection becomes unbalanced in human body, antioxidant supplement may be used to help reduce oxidative damage.

Table.1 Some selected antioxidants and their mechanisms of action[12]:

Antioxidants	**Mechanisms of action**
SOD	Dismutation of superoxide to H2O2
CAT	Decomposes H2O2 to molecular oxygen and water
GSH	Intracellular reducing agent
Lycopene	Trapping of singlet oxygen
Quercetin	H_2O_2 scavenging, one of the potent antioxidant among polyphenols
Vitamin E	Direct scavenging of superoxide

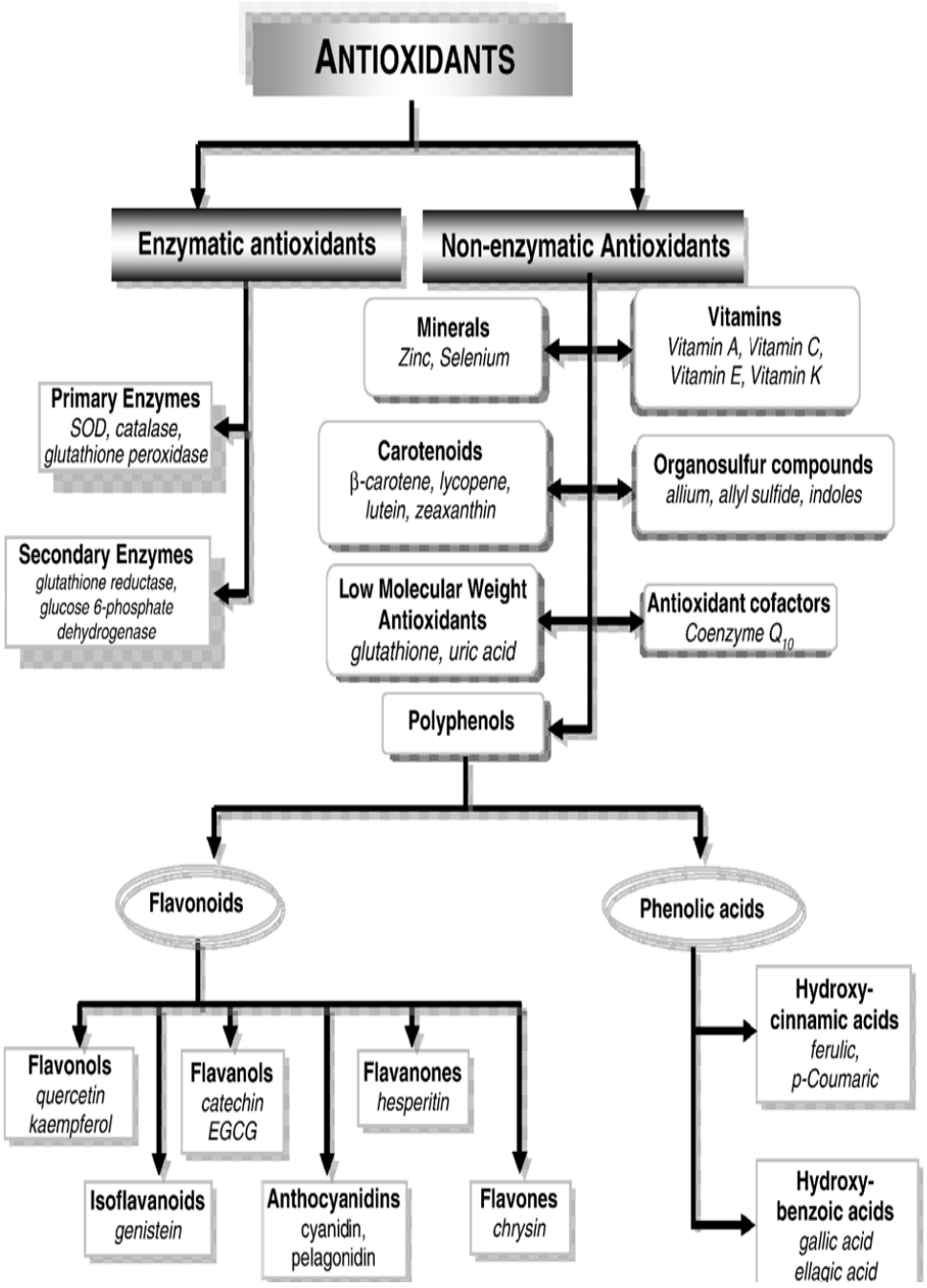


Figure.3 Classification of antioxidants

1.3 FLAVONOIDS:

- Flavonoids are found in higher vascular plants, particularly in the flower, leaves and bark. They are especially abundant in fruits, grains and nuts, particularly in the skins.
- Beverages consisting of plant extracts (beer, tea, wine, fruit juice) are the principle source of dietary flavonoid intake. A glass of red wine has ~200 mg of flavonoids.
- Typical flavonoid intake ranges from 50 to 800 mg/day, which is roughly 5, 50 and 100 times that of Vitamins C, and E, and carotenoids respectively.

1.3.1 Flavonoids role in cancer[13]:

Few diseases are feared more than cancer because cancerous diseases, after cardiovascular disorders and accidents, kill more people before a normal life span has been reached. Besides, the progress of cancer is often accompanied by great pain and ugly disfiguration of the body. Yet, in principle, cancer is curable if it is discovered early and treated with the best current therapeutic methods. However, radical cancer cure is fraught with considerable life-threatening dangers, loss of organs, pain, and discomfort. Besides, its treatment is expensive. Hence, it is understandable that many cancer patients look for and try milder anticancer therapies that offer some promise of a moderate, long-term life-saving cure. The flavonoids are some of the most promising anticancer natural products that have been tried. Related synthetic substances, e.g., flavone acetic acid, have been subjected to Phase I clinical trials already, and they may soon become adopted into the general repertoire of cytostatic treatment.

1.3.2 Flavonoids role in anti oxidants:

a. Enhance or mimic antioxidant enzymes.

b. Direct scavenging of ROS.

c. Repair damaged cellular components.

d. Pro-oxidant metal deactivation

Flavonoid and related compound are effective in scavenging DPPH radical[14], hydroxyl radical and in metal-chelating capacity. Flavonoids are found to exhibit numerous biological activities like vasodilatory, anti carcinogenic, anti-inflammatory, antibacterial, immune-stimulating, antiallergic, and antiviral effects [15].

1.4 CELL LINES[16]:

If the specified number of cells of inoculated in to sensitive mouse strain, tumors can be developed rapidly as compared to chemical carcinogen-induced tumors and time can saved using this model.

1.4.1 METHODS INVOLVED IN CELL LINES:

L-1210 and P-388 cell lines are used. These cell lines are derived from mouse lymphatic leukemia and have 100% growth fraction and tumor implanted with specified number of L-1210 or P-388 cells can be predicted. The effective drug would retard the tumor growth and increase the life span of the animal. A drug, which prolongs the lifespan of the animal by 20%, is taken for subsequent studies involving testing on other transplantable tumors. Some other cell lines which can be inoculated to induce tumors are B-16(melanoma), Lewis lung carcinoma and sarcoma-180, etc. The host mouse strain for above type of cell lines is BDF, except Swiss for sarcoma-180. P-388 and L-1210 cell lines are inoculated intraperitonially and sarcoma-180 as subcutaneously. The experiment takes about 10 days for completion

ILS (%) = [(Mean survival of treated group/ Mean survival of control group)-1] x100

For sarcoma-180 tumors, reduction of tumor size (tumor weight) is used to find out the inhibiting activity of solid tumors as follows.

Tumor inhibiting activity =(average tumor weight of the treated group/average tumor weight of control group) x 100

Hollow-fiber technique:

Small hollow fibers (tubes 1mm in diameter and 2cm long made of plastic, polyvinylidene flouride), containing cells from human tumors are inserted underneath the skin and in the body cavity of the mouse. Each candidate drug is administered at two dosages and is tested against 12 target tumor cells in different hollow fibers. A total of about 20 compounds per week are screened by this method. Compounds that retard the growth of the cells are recommended for the next level of testing. The average length of this test is four days.

Nude mouse model:

Nude mice have been widely used to test the tumorogenicity of cells or for testing of anticancer drugs. These mice are immunologically incompetent because of absence of thymus. They neither show mitotic response in mixed lymphocyte reaction, nor generate cytotoxic effector cell. Lack of helper T and suppressor T cells alters the antibody response of the animals to antigen. They do not show contact sensitivity and do not reject the transplanted material. They are required to be maintained under strictly sterile conditions and in a warm environment (26-280). Some other points regarding their use are:

a. Certain tumors like melanomas and colon carcinomas grow very well in nude mice, whereas prostate carcinoma and most types of leukemia grow very poorly.
b. Large numbers of cells, usually $>10^6$ are required to be inoculated beneath the skin to get a successful tumor take.
c. Metastases are rarely observed.
d. Overall maintenance is very expensive.

1.5 INVIVO METHODS FOR SCREENING OF NEW ANTICANCER MOLECULES[16]:

Studies that are **in vivo** (Latin for "within the living"; often not italicized in English) are those in which the effects of various biological entities are tested on whole, living organisms, usually animals, including humans, and plants as opposed to a partial or dead organism.

USE

- In vivo testing is necessary for medical and research purposes
- The medical field benefits from animal models to test the safety of drugs before they are used on patients
- The research field benefits from in vivo testing by validating in vitro findings in vertebrates closest to humans
- In vivo testing using animal models of disease help discover new ways of solving complex health problems.

METHODS

- Xenograft
- Spontaneous
- Carcinogen-Induced
- GEMs (Genetically Engineered Mouse models)
    - Transgenic
    - Knockout
    - Regulatable transgenic

Xenograft Models of Cancer

- Human cell lines in Immunocompromised mouse
- Human tumor grafts in Immunocompromised mouse
- Mouse cell lines in syngeneic host
- Mouse tumor grafts in syngeneic host.

Subcutaneous Implantation

Implant cells or tissues under the skin.

Advantages

- external monitoring of growth
- easy & most commonly used human cells

Disadvantage

- suboptimal vascular site
- poor take rate
- can be difficult to find tissue that does not grow into a tumor
- can be an irritant to the anima.

Spontaneous Models

Some strains of laboratory animals are susceptible to spontaneously developing certain types of tumors.

Advantages

- May mimic some types of human diseases
- Can use to study early disease
- Can use for prevention
- Includes elements of progression

Disadvantages

- Variability of disease progression
- Large animal numbers needed
- Long time to develop disease
- Penetrance (Not all animals get disease)

Carcinogen-Induced

Animal is treated with a carcinogen to induce cancer.Some laboratory animal Strains are more susceptible.

Examples include:

- Lung cancer
- Skin cancer
- Bladder cancer
- Stomach cancer
- Prostate cancer

Advantages

- Mimics initiation steps of some cancer
- Can study early events
- Used to id predisposing conditions
- Can study prevention

Disadvantages

- Health hazard to investigator
- Variability of disease progression
- Can require large animal numbers
- Penetrance (all animals may not get disease)

Genetically Engineered Mouse (GEM) Models

Simple Transgenic Models

- Tissue specific promoter
- Overexpression of gene
- Ectopic expression (inappropriate expression)
- Oncogene
- Growth factor
- Activated signaling molecules

- Dominant Negative molecules
- Cell cycle regulators Endogenous gene is intact.

Advantages

- May mimic initiation steps of some cancer
- Can study early events
- Can test genetic lesion that predispose
- Autochthonous (rising in the tissue of origin)
- Penetrance usually 100%
- Immune system intact
- Can progress with time

Disadvantages

- Variability of disease progression
- Often requires large animal numbers
- Initiator may be artificial
- Time consuming to characterize & validate
- Expression influenced by site of integration
- May produce chimeric offspring if integrated later in development.

Knockout Models

Approaches:

- Homologous recombination
- Cre-Lox system to KO in a tissue specific manner
- Dominant Negative Transgenic (Functional KO) Disrupts the endogenous gene

Inducible Systems

- Requires a drug or hormone to induce gene expression
- Can be used with Cre to obtain a regulated knockout

Examples include:

- Tet On/Off (Tetracycline)
- RU486 (Progesterone)
- Ecdysone (Insect steroid hormone)

Ideal Inducible System

- Tightly regulated
- Not leaky- low basal level
- Induce to high level of expression
- Induce only in presence of inducer
- Induce nontoxic
- Nonphysiological- not affect expression of endogenous genes
- Reversible expression.

Advantages

- Control when gene is expressed
- Can rescue an embryonic lethal gene
- Allows for normal development of the organ

Disadvantages

- Requires bi-or trigenics to get tissue specific expression
- Large breeding program
- Expensive
- Can be dependent on expression level of regulator

1.6 SIGNIFICANCE OF THE PLANTS CHOSEN FOR THE PRESENT STUDY:

The plant *Plumeria acutifolia* Poir was reported that, it have a rich amount of Flavanoids[18] mainly phenolic compound Gallic acid , Terpenoids, tannins, sterols (β-sitosterol, β-sitosterol-3β -D- glucoside), triterpenes (nonacosane & hentriacontane) and saponins (sapogenins)[19].

1.7 SIGNIFICANCE OF THE DALTON'S LYMPHOMA ASCITES TUMOR CELL LINES (DAL) CHOSEN FOR THE PRESENT STUDY:

1.7.1 Dalton's lymphoma ascites tumor cell lines (DAL):

- ✓ It is a tumor cell line originally grown from a tumor of the thymus.
- ✓ It is propagated by growing as ascites tumor in mice.
- ✓ We can induce both ascites tumor and solid tumors using DAL cells.
- ✓ It is easy to maintain in vivo.
- ✓ It is not an immunogenic.

1.7.2 Maintenance of cell lines:

Tumor Cell Line and Their Maintenance, Dalton's lymphoma ascites tumor cell lines (DLA), originally obtained from Amala Cancer Institute, Thrissur, Kerala were propagated as transplantable tumors in the peritoneal cavity of the mice were used for the study. The tumor cell lines were maintained by serial peritoneal cavity i.p transplantation in mice. Full-grown tumor cell-line were aspirated mouse by injecting PBS in to peritoneal cavity make cells to suspend in PBS, take that suspended solution and count the number of cells present in one ml by using tryphan blue exclusion method and adjust the cell count to 1×106 by using PBS were inject intraperitonially in to a new healthy mouse.

2.REVIEW OF LITERATURE

1. vijaya lakshmi.A, et.al., 2014., reported pharmacognostic phytochemical investigation of the root bark of plant *plumeria acutifolia* showing qualitative and quantitative phytochemical constituents.
2. Akihiko omata et.al., 1992, evaluate the essential oil of *plumeria rubra* forma acutifolia. poir woodson cv. Common yellow growing in hawai was extracted by simultaneous distillationand extraction (SDE).the essential oil was analysed with GC and GC-MS and total 74 compund were identified.
3. A.vijayalakshmi et.al., 2011,studied that the Anti anaphylactic and anti inflammatory activities of a bio active alkaloid from the root bark of *plumeria acutifolia* poir. A dose dependent benificial effect was observed on leakage of evans blue dye in skin changed with antigen and on paw anaphylaxis indused by antiserum .
4. surendra kr sharma, et.al., 2012, assayed Anti microbial potential of *plumeria rubra* syn *plumeria acutifolia* bark were sucessive extracts of *plumeria rubra* syn *plumeria acutifolia* using petrolium ether (60-70°C) chloroform methanol and water. finally reported as plant having anti microbial action.
5. Jeriel naomi B bacer,et al 2017,reported the chemical investigation of dichloro methane extract of the white flower of *plumeria rubra*. L.syn *plumeria acuminata* (W.T.aiton) afforded a mixture of lupeol (1) amyrin (2) amyrin (3) in about 8:2:1 ratio.the structure of 1-3 were identified by comparison of their NMR data with those reported in the literature.
6. Aidroo M.hasant, et.al., 1997, were investigated species of genus plumeria have been investigated for iridoids and triterpenoid and some of these have been found to exhibit algicidal, antibacterial cytotoxic and plant grow inhibiting activity.bioassay result of *plumeria acutifolia* indicate that the ccl_4 extract has antimutagenic activity .
7. nihon yakugakkai. 1988, reported Six new iridoids i.e 13-o caffeoyl plumieride, 13- deoxy plumieride, 13- dihydro plumericinic acid,glucosylester, 1 alpha plumieride , 1 alpha proto plumiericin A, etc were isolated from polar fraction of the methanol percolate of the root of *plumeria acutifolia*.and the presence confirms the plant have antioxidant activity.

8. pradeep kumar R,2014, analysed *Plumeria acutifolia* lactase is used for mouth ulcer were studied in ethonochemical survey on plants used by tribal in chitteri hills.
9. Traditional healing practise in north east india were studied various medicinal plants healing activity.they mentioned their article about *plumeria acutifolia* have antifertility activity.
10. nylane maria nunes de alencar et.al., 2015, were reported *Plumeria rubra* (Apocynaceae) is frequently used in folk medicine for the treatment of gastrointestinal disorders,hepatitis and tracheitis among other infirmities. Investigate the gastroprotective potential of a protein fraction isolated from the latex of *plumeria rubra* (prlb) against ethanol -induced gastric lesions.
11. zahid zaheer et.al., 2010, are studied *Plumeria rubra* used to treat asthma,ease constipation promote mensturation,venerial disease reduce fever and latex used to soothe irritation. The plant contains phyto constituents like cytotoxic iridoids, fulvoplumerin,oleanane type triterpene.
12. umakant sharma, et al 2011,were analysed the genus plumeria apocynaceae is known as a source of iridoids. is this study chloroform extract prepared from the bark of this plant showed significant anti leishmanial activity
13. Gupta M et.al.,2006, reported that the methanol extract of *Plumeria acuminata* leaves exhibited significant anti-inflammatory activity on the tested experimental animal models. Administration of EPA (500 mg/kg b.w) and indomethacin (10 mg/kg b.w) significantly reduced the formation of granuloma tissue induced by cotton pellet method at a rate of 45.06 and 51.57% respectively30. They also studied the anti oxidant activity of the *Plumeria acuminata* leaves[1].
14. Amelia P. Guevara et.al., 2012, were studied anti mutagenic activityof the ethanol extract of the green leaves of *Plumeria acuminata* Air. The antimutagens were isolated from the bioactive hexane and carbon tetrachloride fractions following a bioactivity-directed fractionation scheme and using the micronucleus test to monitor the antimutagenic activities[2].

15. Rasool S. et.al., 2008, studied the Antimicrobial activities of *Plumeria acutifolia* Poir. Ethanolic extract ofstem bark was tested for antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria and fungi by disc diffusion method[3].

3. PLANT PROFILE

***Figure.4 Plumeria acutifolia* Poir**

3.1 BOTANICAL CLASSIFICATION:

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Asteridae
Order	:	Gentianales
Family	:	Apocynacea
Botanical name	:	*Plumeria acutifolia* poir

3.2 COMMON NAMES:

English	:	Temple tree
Hindi	:	Gulachin, Golainchi
Telugu	:	Vaada Ganneru, Deva Ganneru
Tamil	:	Atralari

3.3DESCRIPTION:

Small tree, 3 to 7 m high, stem smooth and shining, succulent, with abundant white latex; easily breaks.

- ✓ Leaves: crowded at the terminal end of the branch, commonly oblong in shape, reaching a length of 40 cm and a width of 7 cm.
- ✓ Flowers: fragrant, the upper portion whitish, while the inner lower portion yellow, 5 – 6 cm long.
- ✓ Fruits: linear-oblong or ellipsoid follicles.

3.4 MEDICINAL USES

- ✓ Decoction of bark is used as purgative.
- ✓ Used in Prevention of heat stroke: the material may be taken as a cooling tea.
- ✓ For dysentery, diarrhea during summer season: use 12 to 24 gms of dried material in decoction.
- ✓ Arthritis, rheumatism, pruritic skin lesions: Mix the latex (sap) with coconut oil, warm, and apply to affected area.
- ✓ Decoction of its bark is used as a counterirritant on the gums for toothache.
- ✓ The latex mixed with coconut oil is used for itching.
- ✓ The juice was used as rubefacient in rheumatic pains, and with camphor, is also used for itching.
- ✓ A poultice of heated leaves is beneficial for swellings.
- ✓ Decoction of leaves used for cracks and eruptions of the soles of the feet.
- ✓ Infusion or extract from leaves is used for asthma.

4.AIM AND OBJECTIVE OF THE STUDY

The aim of the study is to evaluate the Anti Cancer activity. On the world wide basis cancer is the single largest cause of the death both in man and women. Therefore the challenging task at this moment is to identify the quick and novel methods that can identify and develop molecules, which can be of therapeutic value in human cancer.

India has long tradition of the use of drugs, derived from plant sources. Nowadays even the allopathic specialists are also started to move into Ayurvedic, Siddha and Unani system of treatment in western countries, to avoid the untoward effects of certain allopathic medicines, though they are much potent. This indicates that there will be a much better scope for the natural plant sources, which will have a therapeutic value.

The present synthetic cancer drugs produce undesirable side effects and treatment is cost effective. At the same time plant derived anticancer molecules are safer and potent. So in this present study, plant source was selected.

The plants selection in the present study was done on basis of it easy availability and phytochemical constituents to screen their therapeutic potential. The plant *Plumeria acutifolia* Poir contain alkaloids, flavonoids, steroids, phenol and other constituents. Flavonoids and phenol compounds plays main role in free radical scavenging.

Hence an effort was made to explore the exploited properties of the plants, its active constituents and their role in cancer.

5.PLAN OF WORK

Phase I:

5.1. Phytochemical studies:

- Collection and authentication of plants.
- Extraction of plant material by using a suitable solvent system.
- Preliminary phytochemical study for the identification of plant secondary constituents.

Phase II:

5.2. Pharmacological studies:

- Evaluation of anticancer activity of ethanol extract of leaf of *plumeria acutifolia* (EPA) against Dalton's Ascites Lymphoma (DAL) in mice.
- Evaluation of antioxidant enzymes and its parameters with special reference to cancer cell lines.
- Histopathological study of mice liver tissue

Phase III:

5.3 Statistical Analysis

The data's were presented as mean ± SEM and were subjected to statistical analysis by Dunnett's test followed by one way ANOVA. P-value less than 0.05 were considered statistically significant

6.MATERIALS AND METHODS

6.1 MATERIALS

6.1.1 COLLECTION AND AUTHENTICATION OF PLANTS:

The plant materials were collected in the month of July from chinesepet of Salem district and the plants were authenticated by Dr. G.V.S. MURTHY, scientist F, the Botanical Survey of India, Coimbatore.

6.1.2 EXTRACTION PROCESS:

The plants leaves were shade dried and made into coarsely powder. The powder was packed into the soxhlet extractor. The fatty matter was removed by petroleum ether and the active constituents were extracted by using ethanol until the powder gets decolorized. The extract was concentrated by distillation and stored for screening its activity.

6.1.3 ANIMAL USED:

Inbred female Swiss mice of 2 months age, weighing 20 ± 5 g, were purchased from Govt veterinary college Mannuthi, Thrissur, India, were used for the study. The mice were obtained from the stock in breed colony, which was maintained by mating brothers and sisters. They were housed at room temperature of 22°C under 12 hr light/12 hr dark cycle in the animal house. Mice were fed with commercial pellet diet and water *ad libitum* freely throughout the study. All animal procedures were performed after approval from the IAEC (institution of animal ethical committee) and in accordance with the recommendations for the proper care and use of laboratory animals.

6.1.4 DETERMINATION OF ACUTE ORAL TOXICITY

Test Substance Details

Name of the test substance	…..extract
Colour of the test substance	Dark orange
Nature of the test substance	Sticky

Experimental Protocol

Name of the study	Acute toxicity
Guideline followed	OECD 423 method – acute toxic class method
Animals	Healthy young adult Swiss Albino Mice, nulliparous, non-pregnant
Body weight	25-30 g
Sex	Female
Administration of dose and volume	2000 mg/kg body weight, single dose in 0.5 ml
Number of animals	t group animals, second group animals the toxicity sign.
Route of administration	Oral by using mice oral needle
Vehicle	0.2% propylene glycol

Housing and Feeding Conditions

Room temperature	22^{o} C ± 3^{o} C
Humidity	40 – 60 %
Light	12 h :12h (light : dark cycle)
Feed	Standard laboratory animal food pellets with water *ad libitum*

Study Period and Observations Parameters

Initial once observation	First 30 minutes and periodically 24 h
Special attention	First 1–4 h after drug administration
Long term observation	Up to 14 days
Direct observation parameters	Tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.
Additional observation parameters	Skin and fur, eyes and mucous membrane, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern etc.

The time of death, if any, is recorded. (Complete observations: Annexure I). After administration of the drug, food is withheld for a further 1 – 2 hours.

Study Procedure

Acute oral toxicity study was performed as per Organization for Economic Cooperation and Development (OECD) guideline 423 method. The test substance was administered in a single dose by gavage using specially designed mice oral needle. Animals are fasted 3 h prior to dosing (food was withheld for 3 h but not water). Following the period of fasting animals was weighed and test substance was administered. After the test substance administration, food was with held 2 h in mice. Animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hrs, with special attention given during the first 4 hrs, and daily thereafter, for a total of 14 days. Animals are removed, if any humanely killed for animals welfare reasons or are found dead. All the observations are systemically recorded and given in **annexure.**

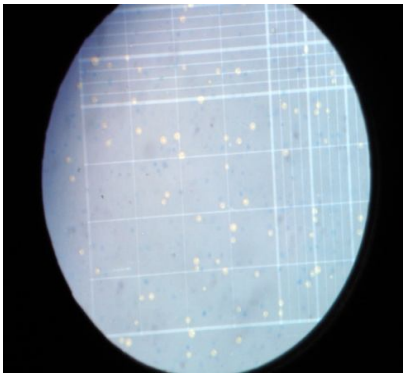


Figure 5.Haemocytometer

6.1.5.EXPERIMENTAL DESIGN

DAL-INDUCED ASCITIC ANTITUMOR MODEL (1)

a) ADJUST CELL COUNT TO 1×10^6 cells:

Requirements:

a) Ehrlich ascites carcinoma (EAC)
b) Phosphate buffer saline solutions

c) Haemocytometer
d) Tryphan blue solution (0.4%)

Method:

0.5ml of 0.4% Tryphan blue, 0.3ml of PBS and 0.2ml of cell suspension were mixed and kept aside for 5min and not more than 15min. From this one drop of solution was taken on a neubar chamber and a cover slip is placed. This is placed on Haemocytometer and the viable and non-viable cells were counted under 10X power. Viable cells doesn't take colour and these cells appear in white colour on blue background Non-viable cells(dead cells) take blue colour and give dark blue shading to the cells, cell count was calculated using formula**.**

Cell count = No. of cells x Dilution factor × volume fact

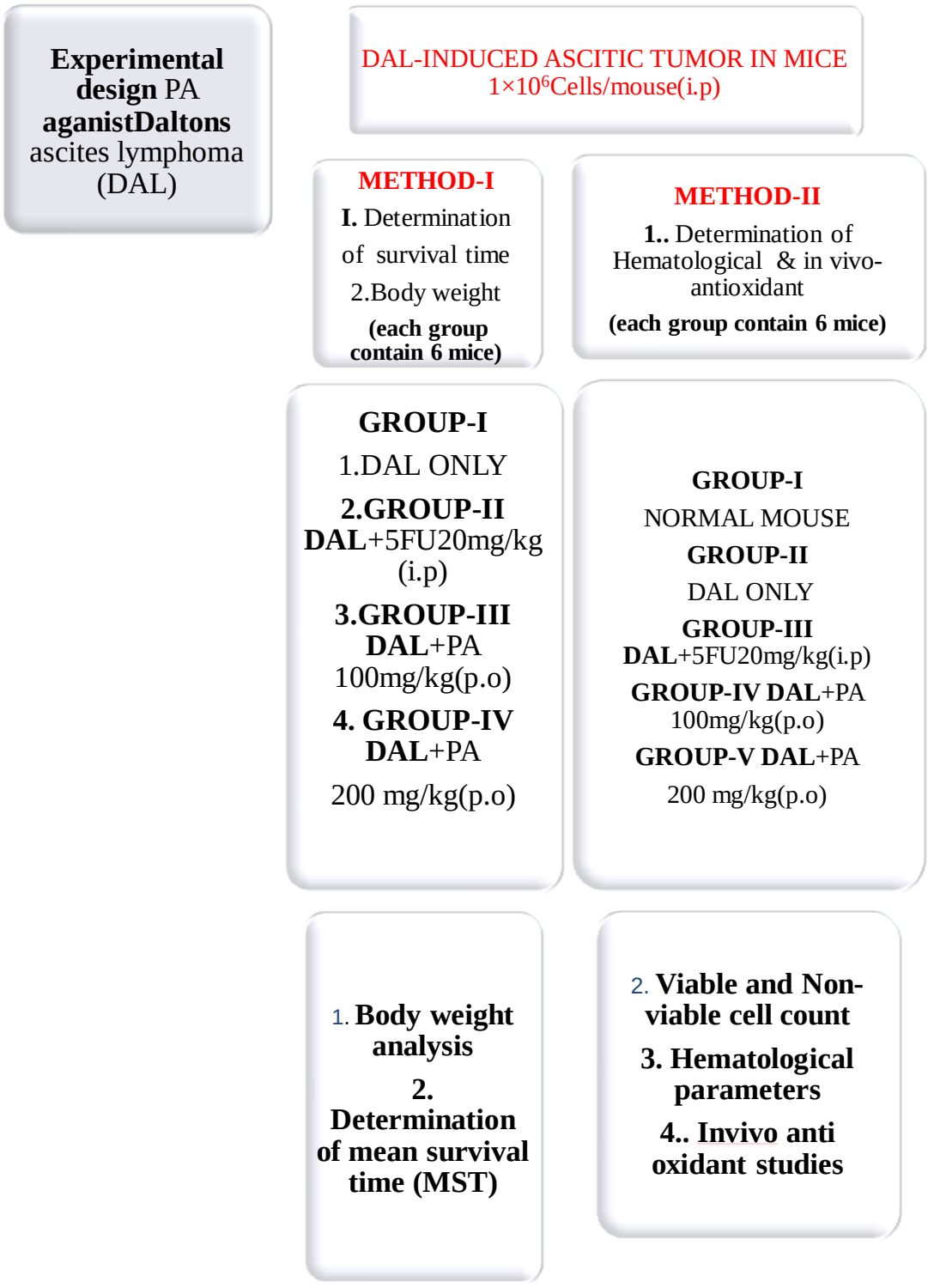


Figure.6 experimental design

b) DAL-induced ascitic antitumor model:

The anti tumor activity of the EXT was determined by injecting DAL cell suspension (1×10^6 cells per mouse) in to the peritoneal cavity of the animals and treatment was started after 24 hours of the tumor inoculation continued once daily for 14 days and the antitumor efficacy of test sample was compared with that of 5-Fu (20mg/kg, i.p) and DAL control.

6.2 METHODS

6.2.1 PRELIMINARY PHYTOCHEMICAL ANALYSIS[39]:

CHEMICAL TESTS:

A) TEST FOR CARBOHYDRATES:

1) **Molisch Test**: In this a small amount of test extract is treated with α-naphthol and concentrated sulphuric acid along the sides of the test tube. Purple colour or reddish violet color at the junction between two liquids was formed. It indicates presence of carbohydrates.
2) **Fehling's Test**: In this small amount of test extract is treated with Equal quantity of Fehling's solution A and B is and Heat gently, brick red precipitate was formed. It indicates presence of carbohydrates.
3) **Benedict's test**: To the 5 ml of Benedict's reagent, added 8 drops of extraction solution. Mixed well, boiling the mixture vigorously for two minutes and then cool. Red precipitate was formed. It indicates presence of carbohydrates.
4) **Barfoed's test**: To the 5 ml of the Barfoed's solution added 0.5 ml of extraction solution and mixed well and heated to boiling, red precipitate was formed. It indicates presence of carbohydrates.

B) TEST FOR ALKALOIDS

1) **Dragendroff's Test**: To the extract, 1 ml of Dragendroff's reagent was added Orange red precipitate was formed. It indicates presence of alkaloids.

2) **Wagner's test**: To the extract Wagner reagent was added. Reddish brown precipitate is formed it indicates presence of alkaloids.
3) **) Mayer's Test**: To the extract added 2 ml of Mayer's reagent. Dull white precipitate was produced. It indicates presence of alkaloids.
4) **Hager's Test**: To the extract added 3 ml of Hager's reagent yellow Precipitate is produced. It indicates presence of alkaloids.

C) TEST FOR STEROIDS AND STEROLS

1) **Libermann Burchard test**: In this test sample is dissolved in 2 ml of chloroform in a dry test tube. Then added10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid. The solution becomes red, then later it was not changed to blue and bluish green colour it indicates absence of steroids and sterols.
2) **Salkowski test**: In this the test sample was dissolved in chloroform and adds equal volume of conc. sulphuric acid. Bluish red cherry red and purple color is not formed in chloroform layer, and also green fluorescence was not formed indicate absence of steroids and sterols.

D) TEST FOR GLYCOSIDES

1) **Legal's test**: The extract Sample is dissolved in pyridine sodium nitropruside solution is added to it and made alkaline. Pink red color is produced. Indicate presence of glycosides.
2) **Baljet test**: To the extract sample, sodium picrate solution is added. Yellow to orange colour is produced. Indicate presence of glycosides.
3) **Borntrager test**: Add a few ml of dilute sulphuric acid to the test solution. Boiled, filtered and extract the filtrate with ether or chloroform. Then organic layer is separated to which ammonia is added, pink, red or violet colour is produced in organic layer. Indicate presence of glycosides.
4) **Killer Killen test**: Sample is dissolved in acetic acid containing trace of ferric chloride and transferred to the surface of concentrated sulphuric acid. At the junction of liquid reddish brown color is produced which gradually becomes blue. Indicate presence of glycosides.

E) TEST FOR SAPONINS

Foam test: About 1 ml of alcoholic sample is diluted separately with distilled water to 20 ml and shacked in graduated cylinder for 15 minutes. 1 cm layer of foam was formed it indicates the presence of saponins.

F) TEST FOR FLAVANOIDS

Shinoda test: To the sample magnesium turnings and then concentrated hydrochloric acid is added. Red color is produced. Indicate the presence of flavanoids.

G) TEST FOR TRITERPENOID

In the test tube, 2 or 3 granules of tin was added, and dissolved in a 2ml of thionyl chloride solution and test solution was added. Pink color is produced which indicates the presence of triterpenoid.

H) TEST FOR PROTEIN AND AMINO ACID

1) **Biuret test**: Added 1 ml of 40% sodium hydroxide and 2 drops of 1% copper sulphate to the extracts, a violet colour formed indicates the presence of proteins.
2) **Ninhydrin test**: Added 2 drops of freshly prepared 0.2% ninhydrin reagent to the extracts and heated. A blue colour developed indicating the presence of proteins, peptides or amino acids.
3) **Xanthoprotein test**: To the extracts, added 20% of sodium hydroxide. Orange colour was formed indicates presence of aromatic amino acid.

6.2.2 DETERMINATION OF BODY WEIGHT AND SURVIVAL TIME

Body weight analysis:

All the mice were weighed for every five days, after tumor inoculation. Average gain in body weight was determined and a % decrease in body weight was calculated by the formula.

%Decrease in body weight = (Decrease in body weight/initial body weight) ×100

Mean Survival Time (MST):

After induction every day checks all the groups for mortality & record how many days the mouse is survived the mean survival time (MST) and percentage increase in life span (ILS %) was calculated by using the formula.

Mean survival time = [1st Death + Last Death] / 2

ILS (%) = [(Mean survival of treated group/ Mean survival of control group)-1] x100

6.2.3 DETERMINATION OF HEMATOLOGICAL & IN VIVO-ANTIOXIDANT

Viable and non-viable cell count:

Requirements:

a) Daltons ascites lymphoma**(DAL)**
b) Phosphate buffer saline solutions
c) Haemocytometer
d) Tryphan blue solution (0.4%)

Method:

After 14 days treatment animals are slightly anaesthetized with diethyl ether from the intraperitonial cavity of mice take 0.2ml of cell suspension were mixed with 0.5ml of 0.4% Tryphan blue, 0.3ml of normal saline or PBS and kept aside for 5min and not more than 15min. From this one drop of solution was taken on a neubar chamber and a cover slip is placed. This is placed on Haemocytometer and the viable and non-viable cells were counted under 10X power Viable cells doesn't take colour and these cells appear in white colour on blue background Non-viable cells(dead cells) take blue colour and give dark blue shading to the cells, cell count was calculated using formula **.**

Cell count = No. of cells x Dilution factor × volume factor

Preparation of Blood Serum and Tissue Samples for The Bio Chemical Studies:

After 14 days treatment the animals were fasted over night and collect the blood sample and intraperitonial fluid by mild anaesthised the mice with diethyl ether and then sacrificed by cervical dislocation. Blood was collected for estimation of RBC, WBC, Hb percentage.

Tissues like liver was removed from the mouse body and tissues were transferred to ice cooled containers. Wiped thoroughly using blotting paper to remove blood and other body fluids then they were washed in normal saline, again wiped desired amounts of dried tissues were used for various biochemical analysis and histopathology studies .

Estimation of Hematological Parameters:

A) Enumeration of white blood cells:2,3

The total white blood cells were enumerated according to the method of John (1972)

Reagents:

Turk's fluid (WBC diluting fluid).

Procedure:

Using a white blood cell pipette of haemocytometer, well mixed blood was drawn up to 0.5 mark and WBC diluting fluid was taken up to mark II. The fluid blood mixture was shaken and transferred onto the counting chamber. The cells were allowed to settle to the bottom of the chamber for 2 min. See the fluid does not get dried.

Using 10X or low power objective the WBC's were counted uniformly in the larger corner squares.

The cells were expressed as number of cells $x10^9/L$

B) Enumeration of red blood cells:

Reagents: RBC diluting fluids

Procedure:

Using a red blood cell pipette of haemocytometer, well mixed blood was drawn up to 0.5 mark and RBC diluting fluid was taken up to mark II. The fluid blood mixture was shaken and transferred onto the counting chamber. The cells were allowed to settle to the bottom of the chamber for 2 min. See the fluid does not get dried. Using 45X or high power objective the RBC's were counted uniformly in the larger corner squares.

The cells were expressed as number of cells $x10^{12}$/L or x 10^{6} / cu.mm

C) Differential Leukocyte Count:

Differential Leukocyte count was determined by the method of John (1972).

Reagent:

Leishmann's stain: 150mg of powdered leishmann's stain was dissolved in 133ml of acetone free methanol.

Procedure:

A blood film stained with leishmann's stain was examined under oil immersion and the different types of WBCs were identified. The percentage distribution of these cells was then determined. Smears were made from anticoagulant blood specimens and stained with leishmann's stain. The slides were preserved for counting the number of lymphocytes and neutrophils, per 100 cells were noted.

The number of neutrophils was expressed as (%)

D) Estimation of Hemoglobin:

Sahli's acid haematin method:

Principle:

Haemoglobin is converted into acid haematin by the action of HCl. The acid haematin solution is further diluted with distilled water until its colour matches with exactly that of permanent standard of comparator block. The Hb concentration is read directly from the calibration tube.

Requirements:

HCl solution, sahli's Hemoglobinometer, pipette, distilled water.

Procedure:

By using pipette add 0.1 N HCl in the Hemoglobinometer up to the lowest marking. 20µl of blood was drawn up to 20µl in the sahli's pipette. Adjusted the blood column carefully without bubbles. Wiped the excess of blood on the sides of the pipette by using a dry piece of cotton. Blown the blood into the acid solution in the graduated tube, rinsed the pipette well. Mixed the reaction and allow the mixture to stand at room temperature of 10 minutes. Diluted the solution with distilled water by adding few drops of water carefully and by mixing the reaction mixture until the colour matches the colour in the comparator. The lower meniscus of the fluid was noted and reading was noted in g/100ml. [4.]

6.2.4 SERUM BIO CHEMICAL PARAMETRES

The separated serum was used for estimation of alkaline phosphatase ALP), Serum glutamic oxaloacetic transaminase SGOT) and serum glutamic pyruvic transaminase SGPT), serum creatinine and protein by using semi auto analyzer Photometer 5010 v_{5+})using standard enzymatic kits procured from Piramal Healthcare limited, Lab Diagnostic Division, Mumbai, India.

❖ **ESTIMATION OF SGOT:** Serum glutamate oxalo acetate transaminase (SGOT)

Principle:

L-Aspartate + 2-Oxaloglutarate $\xleftrightarrow{ASAT}$ L-Glutamate + Oxaloacetate

Oxaloacetate + NADH + H^+ $\xleftrightarrow{MDH}$ D-Malate + NAD^+

Addition of pyridoxal-5 phosphate P-5-P) stabilizes the transaminases and avoids falsely low values in samples containing insufficient endogenous P-5-P, e.g. From patients with myocardial infraction, liver diseases and intensive care patients.

Table.3 Reagents

R 1	TRIS pH 7.8	80 mmol/l
	L- Aspartate	240 mmol/l
	MDH malate dehydrogenase)	≥600 U/l
	LDH lactate dehydrogenase)	≥600 U/l
R 2	2-Oxaloglutarate	12 mmol/l
	NADH	0.18 mmol/l
	Pyridoxal-5-phosphate FS	
	Good buffer pH 9.6	0.7 mmol/l
	Pyridoxal-5-phosphate	0.9 mmol/l

Method:

Optimized UV- test according to IFCC International Federation Of Clinical Chemistry and Laboratory Medicine).

Assay procedure:

a) Mixed 800 µl of reagent-1 with 200 µl of reagent-2 in a 5 ml test tube.

b) To this, added 100 µl of serum.

c) Mixed well and took the reading immediately.

Clinical significance:

AST Occurs in all human tissue and is present in large amounts in liver, renal cardiac and skeletal muscle tissue.

Increases:

increased levels are associated with liver disease or damage, myocardial infarction, cholecystitis.

Decreases:

decreased levels are observed in patient undergoing renal dialysis and those with vitamin B 6 deficiency.

Reagent composition:

when reconstituted as directed)

Normal Range: < 41 U/l

❖ **ESTIMATION OF SGPT:**

Serum glutamate pyruvate transaminase (SGPT):

Principle:

L-Alanine +2-oxoglutarate $\xleftrightarrow{\text{ALAT}}$ L-Glutamate + Pyruvate

Pyruvate + NADH + H^+ $\xleftrightarrow{\text{LDH}}$ D-Lactate + NAD^+

Addition of pyridoxal-5-phosphate P-5-P) stabilizes the transaminases and avoids falsely low values in samples containing insufficient endogenous P-5-

P,eg.from patients with myocardial infarction, liver diseases and intensive care patients.

Method:

Kinetic UV test, according to the International federation of clinical chemistry and laboratory medicine IFCC).

Table.4 Reagents

R 1	TRIS pH 7.5	100 mmol/l
	L- Alanine	500 mmol/l
	LDH lactate dehydrogenase)	≥ 1200 U/l
R 2	2-Oxoglutarate	15 mmol/l
	NADH	0.18 mmol
	Pyridoxal-5-phosphate FS	
	Good buffer pH 9.6	0.7 mmol/l
	Pyridoxal-5-phosphate	0.9 mmol/l

Normal range: < 41 u/l

Clinical significance:

ALT is present in high concentration in liver and to a lesser extent in kidney, heart, skeletal muscle, pancreases, spleen and lungs.

Increases:

Increased levels are generally resulting of primary liver disease such as cirrhosis's carcinoma, viral or obstructive jaundice.

Decreases:

decreased levels may be observed in renal dialysis patients and that vitamin B6 deficiency.

❖ **ESTIMATION OF SERUM ALKALINE PHOSPHATASE (ALP):**

Alkaline phosphatase (ALP), and hydrolytic enzyme act optimally at an alkaline pH. They are present in blood in numerous distinct forms which originate mainly from bone and liver

p-Nitro phenyl phosphate +water $\xrightarrow{\text{ALP}}$ Phosphate + *p*-Nitro phenol

Kinetic photometric test, according to the international Federation of clinical chemistry and laboratory Medicine IFCC)

Table.5 Reagents

Reagent 1 :	**Concentration**
2-Amino-2-methyl-1-propanol pH 10.4	0.35 mol/l
Magnesium sulphate	2.0 mmol/l
Zinc sulphate	1.0mmol/l
HEDTA	2.0 mmol/l
Reagent 2 :	Concentration
p-Nitrophenylphosphate	16.0mmol/l

Assay method:

1. Taken 1000 µl of reagent-1 in a 5 ml test tube
2. To this added 250 µl of reagent-2 and mixed well

3. Add 20 µl of serum and mix well and take reading immediately using a photometer.
4. Normal range: 53-128 µ/l. **Soldin *et.al.*, 1996)**

Principle:

Under alkaline condition, colorless p-nitrophenol is converted to 4 nitrophenoxide, which develops a very intense yellow color. Its intensity is proportional to the activity of alkaline phosphatase in the sample.

P-Nitro-phenylphosphate + H_2O $\xrightarrow{ALP>}$ p-Nitrophenol + Phosphate

Clinical Significance :

ALP is present in high concentrations in liver, bone, placenta intestine and certain tumors Increases

Normal:

Physiologically elevated serum alkaline phosphatase occurs in pregnant women and in pregnant women and in growing children.

Pathological:

Increased levels of the enzyme occur in liver diseases, bone diseases Rickets, Paget's Disease), Hodgkin's disease or congestive heart failure.

Decreases :

Decreased levels occur in hypophosphatasia and malnourished patients.

❖ **ESTIMATION OF SERUM BILIRUBIN:**

Principle :

Bilirubin reacts with diazotized sulphanilic acid in acidic medium to form pink colored azobilirubin with absorbance directly proportional to Bilirubin concentration. Direct Bilirubin, being water soluble directly reacts in acidic medium. However indirect or unconjugated Bilirubin is solubilised using a surfactant and then it reacts similar to Direct Bilirubin.

Clinical Significance:

Bilirubin is a breakdown product of haemoglobin. Bilirubin formed in the reticulo-endothelial system is transported to the liver bound to albumin. This bilirubin is water insoluble and is known as indirect or unconjugated bilirubin. In the liver, bilirubin is conjugated to glucuronic acid to form direct bilirubin. Conjugated bilirubin is excreted via the bilary system into the intestine where it is metabolized by bacteria to urobilinogen and stercobilinogen.

Total Bilirubin = Indirect Bilirubin + Direct Bilirubin

Increases :

Total Bilirubin is elevated in obstructive conditions of the bile duct, hepatitis, cirrhosis, in hemolytic disorders and several inherited enzyme deficiencies.

Indirect Bilirubin is elevated by pre-hepatic causes such as hemolytic disorders or liver diseases resulting in impaired entry, transport or conjugation within the liver. Monitoring of direct bilirubin in neonates is of special importance as it is the indirect or free) bilirubin bound to albumin that is able to cross the blood brain barrier more easily, increasing the danger of cerebral damage.

Table.6 Reagent Composition :

Reagent 1: Total Bilirubin Reagent

Surfactant	1.00%
HCl	100 m mol/L
Sulphanilic Acid	5 m mol/L

Reagent 2: Direct Bilirubin Reagent

Sulphanilic Acid	10 m mol/L
HCl	100 m mol/L

Reagent 3: Sodium Nitrite Reagent

Sodium Nitrite	144 m mol/L

Assay Procedure: Allow the working reagent to attain 37 c performing the test.

Pipette into test tubes marked	**Blank**	**Standard**	**Test**
Working Reagent	500 µl	500 µl	500 µl
Distilled Water	25 µl	-	-
Standard / Calibrator	-	25 µl	-
Test	-	-	25 µl

Mix well; incubate for 5 minutes at 37°C for Total Bilirubin and Direct Bilirubin. Read Absorbance at 546/630 nm against reagent blank.

❖ ESTIMATION OF TOTAL PROTEIN

Estimation of Protein:

Protein was estimated by the method of Lowry *et al.* 1951).

Reagents:

1. Alkaline copper reagent

   Solution A: 2% sodium carbonate in 0.1 N NaOH

   Solution B: 0.5% copper sulphate in 1% sodium potassium tartarate

   50 ml of solution A was mixed with 1 ml of solution B just before use.
2. Folin's phenol reagent commercial reagent, 1:2 dilution)
3. Bovine serum albumin (BSA).

Procedure:

To 0.1 ml of suitably diluted plasma/homogenate/hemolysate, 0.9 ml of water and 4.5 ml of alkaline copper reagent were added and kept at room temperature for 10 min. Then 0.5 ml of Folin's reagent was added and the colour developed was read after 20 min at 640 nm. The level of protein was expressed as mg/g tissue or mg/dl.

Normal Range: 6.6-8.7 gm/dl

Determination of biochemical parameters

For assessment of liver function, blood samples were collected from the animals by puncturing the retro-orbital plexus and centrifuged. The serum collected after centrifugation was analyzed for various biochemical parameters like SGOT, SGPT, ALP, TB and total protein (TP) (Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, 1951.Protein measurement with the Folin phenol *reagent*. J Biol Chem 193: 265.). Serum transaminase activity was measured according to the method of Rietman and Frankle. (Reitmann S, Frankel S, 1957.

A colorimetric method for the determination of serum oxaloacetic and glutamic pyruvate transminases. American Journal of Clinical Pathology.28: 56-63). The ALP and the serum bilirubin was determined by using method of Kind. (Kind PRM, King EJ, 1972. *In-vitro* determination of serum alkaline phosphatase. Journal of Clinical Pathology 7: 321-22.)

Determination of aspartate aminotransferase (AST)

Aspartate aminotransferase, also known as Glutamate Oxaloacetate Transaminase (GOT) catalyses the transamination of L-aspartate and α keto glutarate to form oxaloacetate and L- glutamate. Oxaloacetate formed is coupled with 2,4-Dinitrophenyl hydrazine to form hydrazone, a brown coloured complex in alkaline medium which can be measured colorimetrically.

Reagents:

Buffered aspartate (pH 7.4); 2,4- DNPH reagent; 4N sodium hydroxide; working pyruvate standard; solution I (prepared by diluting 1 ml of reagent 3 to 10 ml with purified water).

Procedure

Rietman and Frankle method was adopted for the estimation of SGOT. (Reitmann S, Frankel S, 1957. A colorimetric method for the determination of serum oxaloacetic and glutamic pyruvate transminases. American Journal of Clinical Pathology.28: 56-63.The reaction systems used for this study included blank, standard, test (for each serum sample) and control (for each serum sample). 0.25 ml of buffered aspartate was added into all the test tubes. Then 0.05 ml of serum was added to the test group tubes and 0.05 ml of working pyruvate standard into the standard tubes. After proper mixing, all the tubes were kept for incubation at 37^{o}C for 60 min, after which 0.25 ml each of 2,4- DNPH reagent was added into all the tubes. Then, 0.05 ml of distilled water and 0.05 ml of each serum sample was added to the blank and the serum control tubes respectively. The mixture was allowed to stand at room temperature for 20 min. After incubation, 2.5 ml of solution I was added to all test tubes. Mixed properly and optical density was measured in a spectrophotometer at 505 nm within 15 min.

The enzyme activity was calculated as:-

AST (GOT) activity in IU/L) = [(Absorbance of test - Absorbance of control)/ (Absorbance of standard - Absorbance of blank)] x concentration of the standard

Determination of alanine aminotransferase (ALT)

Alanine aminotransferase, also known as Glutathione Peroxidase (GPT) catalyses the transamination of L-alanine and α keto glutarate to form pyruvate and L-Glutamate. Pyruvate so formed is coupled with 2,4 – Dinitrophenyl hydrazine to form a corresponding hydrazone, a brown coloured complex in alkaline medium which can be measured colorimetrically.

Reagents

Buffered alanine (pH 7.4), 2,4–DNPH, 4N sodium hydroxide, working pyruvate standard, solution I (prepared by diluting 1 ml of reagent 3 to 10 ml with purified water).

Procedure

Rietman and Frankle method was dopted for the estimation of SGPT. The reaction systems used for this study included blank, standard, test (for each serum sample) and control (for each serum sample). 0.25 ml of buffered alanine was added into all the test tubes. This was followed by the addition of 0.05 ml of serum into the test group tubes and 0.05 ml of working pyruvate standard into the standard tubes. After proper mixing, all the tubes were kept for incubation at 37^{o}C for 60 minutes, after which 0.25 ml each of 2,4- DNPH reagent was added into all the tubes. Then, 0.05 ml of distilled water and 0.05 ml of each serum sample was added to the blank and the serum control tubes respectively. The mixture was allowed to stand at room temperature for 20 min. After incubation, 2.5 ml of solution **I** was added to all test tubes. Mixed properly and optical density was read against purified water in a spectrophotometer at 505 nm within 15 min.

The enzyme activity was calculated as:- ALT (GPT) activity in IU/L) = [(Absorbance of test - Absorbance of control)/ (Absorbance of standard - Absorbance of blank)] x concentration of the standard.

Determination of alkaline phosphatase (ALP)

Alkaline phoshatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. Phenol so formed reacts in alkaline medium with 4-aminoantipyrine in presence of the oxidising agent potassium ferricyanide and forms an orange-red coloured complex, which can be measured spectrometrically. The color intensity is proportional to the enzyme activity.

Reagents:

- Buffered substrate
- Chromogen Reagent
- Phenol Standard, 10 mg%

Procedure:

ALP was determined using the method of Kind (Kind PRM, King EJ, 1972. *In-vitro* determination of serum alkaline phosphatase. Journal of Clinical Pathology 7: 321-22\). The working solution was prepared by reconstituting one vial of buffered substrate with 2.2 ml of water. 0.5 ml of working buffered substrate and 1.5 ml of purified water was dispensed to blank, standard, control and test. Mixed well and incubated at 37^0C for 3 min. 0.05 ml each of serum and phenol standard were added to test and standard test tubes respectively. Mixed well and incubated for 15 min at 37^0C. Thereafter, 1 ml of chromogen reagent was added to all the test tubes. Then, added 0.05 ml of serum to control. Mixed well after addition of each reagent and the O.D of blank, standard, control and test were read against purified water at 510 nm.

Serum alkaline phosphatase activity in KA units was calculated as follows [(O.D. Test-O.D. Control) / (O.D. Standard- O.D. Blank)] x 10

Determination of bilirubin

In toxic liver, bilirubin levels are elevated. Hyperbilirubinemia can result from impaired hepatic uptake of unconjugated bilirubin, such a situation can occur in generalized liver cell injury, certain drugs (e.g Rifampin and probenecid) interfere with the rat uptake of bilirubin by the liver cell and may produce a mild unconjugated hyperbilirubinemia. Bilirubin level rises in diseases of hepatocytes, obstruction to bilirubin excretion into duodenum, in haemolysis and defects of hepatic uptake and conjugation of Bilirubin pigment such as Gilbert's disease.

Elevation of total serum bilirubin may occur due to:

1. Excessive haemolysis or destruction of the red blood cells.Eg:Haemolytic disease of the new born.
2. Liver diseases.Eg.Hepatitis and cirrhosis.
3. Obstruction of the biliary tract.Eg.Gall stones.

The method is based on the reaction of Sulfonilic acid with sodium nitrite to form azobilirubin which has maximum absorbance at 546nm in the aqueous solution. The intensity of the color Produced is directly proportional to the amount of direct or total bilurubin concentration present in the sample.

Reagents

1. Diazo A-(Reagent-R1) :Ready to use
2. Diazo B-(Reagent-R2):Ready to use
3. Bilirubin Activater :Ready to use

Procedure

Kind & King's method was followed for the estimation of Bilirubin. Five hundred µl of working reagent was added to 50 µl of rat serum & incubated for 5 min at 37°C. Absorbance was measured AT 546 NM in semi auto analyzer against the standard.

The Bilirubin content was calculated using the following equation:

Total bilirubin (mg/dt) = Abs of the sample blank x 15.

Direct Bilirubin(mg/dt) = Abs of sample blank x 10.

6.2.5.DETERMINATION OF ANTIOXIDANT ENZYMES AND LIPID PEROXIDATION

Preparation of tissue homogenate

After treatment with ethanol extracts of both the plants, the rats were sacrificed, Kidney and liver of rats were isolated and washed with normal saline and stored for 12 h for in vivo antioxidant studies. The separated liver and kidneys were homogenized with motor driven Teflon coated homogenizer with 0.1 M Tris-HCl buffer (pH 7.4) to get 10% homogenate. The homogenate was centrifuged at 10000 rpm for 10 min at 5˚C. The supernatant was collected and used for in vivo studies.

Antioxidant enzymes viz. Superoxide dismutase (SOD)[31], Catalase (CAT)[32], Glutathione peroxidise (GPX)[33], Reduced glutathione (GSH)[34] and Lipid peroxidation (LPO)[35] were determined in all the liver tissues of all the tested rats

Determination of proteins

This method is a combination of both Folin-ciocalteau and Biuret reaction which involves two step reaction. In the first Step Protein binds with copper in

alkaline medium and reduces it to Cu++. In the second step Cu++ formed catalyses the oxidation reaction of aromatic amino acid by reducing Phosphomolybdotungstate to heteropolymolybdanum, which leads to the formation of blue colour which is measured at 640 nm.

Reagents

- Alkaline copper reagent
- Solution A: 2 % w/v of sodium carbonate in 0.1 N NaOH.
- Solution B: 0.5 % w/v copper sulphate in 1 % sodium potassium tartarate 50 ml of
- solution A was mixed with 1 ml of solution B just before use.
- Folin's phenol reagent commercial reagent, 1:2 dilutions) bovine serum albumin (BSA)

Procedure

Lowry method was adopted for the estimation of total protein. (Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, 1951. Protein measurement with the Folin phenol *reagent.* J Biol Chem 193: 265).To 0.1 ml of the liver homogenate, 0.9 ml of water, 4.5 ml of alkaline copper sulphate reagent were added and allowed to stand in the room temperature for 10 min. To this 0.5 ml of Folin's reagent was added. After 20 min, the blue colour developed was measured at 640 nm. The level of protein present was expressed as mg/g tissue or mg/dl.

Determination of superoxide dismutase

Superoxide dismutase scavenges the superoxide radical ($O^{\bullet}_2$) and thus provides a first line defence against free radical damage. Superoxide dismutase is an endogenous enzymatic antioxidant which catalyzes the dismutation of superoxide free

radical. This method is based on the inhibition of the spontaneous oxidation of the adrenaline to adrenochrome by the enzyme superoxide dismutase.

$$2H_2O + 2O_2^- \xrightarrow{\text{Superoxide dismutase}} 2H_2O_2 + O_2$$

Superoxide anion (O_2^-) interacts with peroxide to form hydroxyl radical ($OH^\bullet$) which causes damage in the absence of superoxide dismutase activity ($R^\bullet$)

Reagents

Carbonate buffer – 0.05M, pH 10.2: 1.14 g of sodium carbonate and 84 g of sodium bicarbonate were dissolved in 100 ml of distilled water.

Ethylene diamine tetra acetate – 0.49M: 14.3 g of EDTA was dissolved in 100 ml of distilled water.

Epinephrine – 3M: 54 mg of epinephrine was dissolved in 100 ml of distilled water.

Procedure

SOD was estimated as per the procedure described by Kakkar et al. (**Kakkar P. Das,**. B. and Viswanathan .P.N. A modified spectrophotometic assay of SOD, *Ind. J. Biochem. Biophy.* 1984:21:130-132.). Liver homogenate (0.5 ml) was diluted with 0.5 ml of distilled water. To this, 0.25 ml ethanol and 0.15 ml of chloroform, all reagents chilled, were added. The mixture was shaken for 1 minute and centrifuged at 2000 rpm. The enzyme in the supernatant was determined. To 0.5 ml of the supernatant, 1.5 ml of buffer was added. The reaction was initiated by the addition of 0.4 ml epinephrine and change in optical density per minute was measured at 480 nm in a double beam UV-VIS spectrophotometer (UV 1700,Szhimadzhu)

SOD activity was expressed as U/mg. Change in optical density per minute at 50% inhibition to adrenochrome transition by the enzyme is taken as one enzyme unit.

Determination of catalase

In animals, catalase is present in all major body organs, especially being concentrated in liver and erythrocyte. During β-oxidation of fatty acids by

flavoprotien dehydrogenase, hydrogen peroxide is generated, which is accepted upon by Catalase present in peroxisomes.

Catalase catalyses the rapid decomposition of hydrogen peroxide to water.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Dichromate in acetic acid was converted to perchloric acid and then to chromic acetate when heated in presence of hydrogen peroxide. The chromic acetate thus produced is measured spectrophotometrically at 610 nm. The reaction is stopped at specific time interval by the addition of dichromate- acetic acid mixture and the remaining hydrogen peroxide is determined by measuring chromic acetate.

Reagents

Dichromate-acetic acid reagent: Five % potassium dichromate was prepared with acetic acid (1:3 v/v in distilled water).

Phosphate buffer - 0.01M, pH 7.0: 173 mg of disodium hydrogen phosphate and 122 mg of sodium dihydrogen phosphate were dissolved in 200 ml of distilled water.

Hydrogen peroxide – 0.2M: 2.27 ml of hydrogen peroxide was made upto 100 ml with distilled water.

Procedure

The catalase activity was assayed by the method of Sinha (1972) **(Sinha, A.K**. Colorimetric assay off catalase *Anal* . *Biochem*. 1972: 47: 389-394). Liver homogenate (0.1 ml) was taken, to which 1.0 ml of phosphate buffer and hydrogen peroxide were added. The reaction was arrested by the addition of 0.2 ml dichromate acetic acid reagent. Standard hydrogen peroxide in the range of 4 to 20 µl were taken and treated similarly. The tubes were heated in a boiling water bath for 10 min. The green color developed was read at 570 nm in a Double beam UV-VIS spectrophotometer (UV 1700,Szhimadzhu).Catalase activity was expressed as U/mg.

Determination of glutathione peroxidase

Glutathione peroxidase catalyses the following reaction.

$$R\text{-}COOH + 2GSH + O_2 \xrightarrow{\text{Glutathione peroxide}} R\text{-}CH(OH)\text{-}COOH + GSSG + H_2O$$

Glutathione was measured by its reaction with DTNB to give a compound that absorbs at 412 nm.

Reagents

- Sodium phosphate buffer - 0.32M, pH 7.0: 6.96 g of disodium hydrogen phosphate and 3.89 g of sodium dihydrogen phosphate was dissolved in 200 ml of distilled water.
- Ethylene diamine tetra acetate (EDTA) - 0.8 mM: 233 mg of EDTA was dissolved in 100 ml of distilled water.
- Sodium azide-10mM: 6.5 mg of sodium azide was dissolved in 100 ml of distilled water.
- Reduced glutathione - 4 mM: 122 mg of glutathione was dissolved in 100 ml of distilled water.
- Hydrogen peroxide - 2.5 mM: 0.03 ml of H_2O_2 was made up to 100 ml with distilled water.
- Trichloro acetic acid - 10%: 10 g of TCA was dissolved in 100 ml of distilled water.
- Disodium hydrogen phosphate - 0.3 M: 4.25 g of disodium hydrogen phosphate was dissolved in 100 ml of distilled water.
- DTNB: 40 mg of 5,5'-dithio bis (2-nitrobenzoic acid) was dissolved in 100 ml of 1% w/v sodium citrate.
- Reduced glutathione standard: 20 mg of reduced glutathione was dissolved in 100 ml of distilled water.

Procedure

The glutathione peroxidase activity was measured according to the method of Rotruck *et al.,* (1973) (**Rotruck. J.T**. pope , A.L. , Ganther , H.E. , Swanson , A.B.,

Hateman., D.G.., and Hoekstra , W.G., Selenium, biochemical roles as a component of glutathione peroxidase . *J. Science*, 1973: 179: 588-590.)

EDTA (0.2 ml each), sodium azide, reduced glutathione, H_2O_2; 0.4 ml of buffer and 0.1 ml of enzyme (liver homogenate) were mixed and incubated at 37°C for 10 min. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged. To 0.5 ml of supernatant, 3.ml of sodium hydrogen phosphate and 1 ml of DTNB were added and the color developed was read at 412 nm immediately in a Double beam UV-VIS spectrophotometer (UV 1700,Szhimadzhu.Glutathione peroxidase activity, in serum is expressed as μg/mg.

Determination of reduced glutathione

DTNB (5, 5'-dithiobis (2-nitrobenzoic acid)), known as Ellman's Reagent, was used for the detection of thiol compounds. DTNB and glutathione (GSH) react to generate 2-nitro-5-thiobenzoic acid and glutathione disulfide (GSSG), where 2-nitro-5-thiobenzoic acid yield a stable yellow colored product, which is proportional to GSH concentration, measured at 412 nm.

Reagents

- 10% TCA
- 0.6 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 0.2 M sodium phosphate
- 0.2 M Phosphate buffer, pH 8.0

Procedure

Reduced Glutathione was estimated by Ellman's procedure.(Ellman G.L: Tissue sulphydryl groups: Archives in biochemistry and biophysics: 1959: vol 82 (1):pp 70-77).To 250 μL of tissue homogenate taken in 2 ml eppendroff tube, 1 mL of 5% TCA was added and the above solution was centrifuged at 3000 g for 10 min at room temperature. To 250 μL of the above supernatant, 1.5 ml of 0.2 M phosphate buffer was added and mixed well. 250 μL of 0.6 mM of Ellman's reagent (DTNB solution) was added to the above mixture and the absorbance was measured at 412 nm within 10 min. A standard graph was plotted using glutathione reduced solution (1

mg/ml) and GSH content present in the tissue homogenates was calculated by interpolation. Amount of glutathione expressed as μg/mg protein.

Determination of lipid peroxidation

In this method malondialdehyde and other TBARS were estimated by their reactivity with thiobarbituric acid TBA) in acidic condition to generate a pink coloured chromophore which were read at 535 nm.

Reagents:

- TCA-TBA-HCl reagent: 15% w/v TCA, 0.375 w/v TBA and 0.25 N HCl. The solution was heated mildly to assist the dissolution of the TBA.
- 0.25 N HCl
- 15% TCA

Procedure

Lipid peroxidation was estimated by the method of Okhawa *et al*., (1979)(Okhawa, H, Ohishi, N and Yagi, Assay for lipid peroxidase in animal tissue by thiobarbituric acid reaction anal. *Biochem,* 1979:95; 351 – 8). one ml of liver homogenate was mixed with 0.2 ml 4 % (w/v) sodium dodecyl sulfate, 1.5 ml 20% acetic acid in 0.27 M hydrochloric acid (pH 3.5) and 15 ml of 0.8% thiobarbituric acid (TBA, pH 7.4). The mixture was heated in a hot water bath at 85˚C for 1 h. The intensity of the pink colour developed was read against a reagent blank at 532 nm following centrifugation at 1200 g for 10 min. The concentration was expressed as *n* moles of MDA per mg of protein using 1,1,3,3,-tetra-ethoxypropane as the standard. The results were tabulat

Estimation of ascorbic acid (vitamin C)

The level of ascorbic acid was estimated by the method of Omaye *et al.* 1979).

Reagents

- 5% TCA
- DTC reagent 3 g of 2,4-dinitrophenyl hydrazine, 0.4 g of thiourea and 0.05 g of copper sulphate were dissolved in 100 ml of 9 N sulphuric acid)
- 65% sulphuric acid
- Ascorbic acid

Procedure

To 0.5 ml of homogenate, 0.5 ml of water and 1 ml of TCA were added, mixed thoroughly and centrifuged. To 1 ml of the supernatant, 0.2 ml of DTC reagent was added and incubated at 37°C for 3 hrs. Then 1.5 ml of sulphuric acid was added, mixed well and the solutions were allowed to stand at room temperature for another 30 min. The colour developed was read at 520 nm.

The level of ascorbic acid was expressed as μg/mg protein.

Estimation of vitamin E

The level of vitamin E was estimated by the method of Desai 1984).

Reagents

1. Ethanol
2. Petroleum ether
3. 0.2% 4,6-diphenyl-1,10-phenanthroline in ethanol
4. 0.001 M Ferric chloride in ethanol
5. 0.001 M o-phosphoric acid in ethanol
6. α-Tocopherol acetate

Procedure

To 1 ml of homogenate, 1 ml of ethanol was added and thoroughly mixed. Then 3 ml of petroleum ether was added, shaken rapidly and centrifuged. 2 ml of supernatant

was taken and evaporated to dryness. To this 0.2 ml of bathophenanthroline was added. The assay mixture was protected from light and 0.2 ml of ferric chloride was added followed by 0.2 ml of o-phosphoric acid. The total volume was made up to 3 ml with ethanol. The colour developed was read at 530 nm.

The level of vitamin E was expressed as μg/mg protein.

6.2.6 Statistical Analysis:

The data for various biochemical parameters were analyzed using analysis of variance ANOVA) followed by Tukey multi comparison of all pairs of column.

6.2.7 HISTOPHATOLOGICAL TECHINIQUES:

Histopathology is the microscopical study of tissues for pathological alterations. This involves collection of morbid tissues from biopsy or necropsy, fixation, preparation of sections, staining and microscopical examination.

Collection of materials:

Thin pieces of 3 to 5 mm, thickness are collected from tissues showing gross morbid changes along with normal tissue.

Fixation:

Keeping the tissue in fixative for 24-48 hours at room temperature

a) Serves to harden the tissues by coagulating the cell protein,
b) Prevents autolysis,
c) Preserves the structure of the tissue, and
d) Prevents shrinkage: The volume of the fixative added is 10times the volume of the tissues.

   Common Fixatives: 10% Formalin

Haematoxylin and eosin method of staining:

Deparaffinise the section by xylol 5 to 10 minutes and remove xylol by absolute alcohol, then wash in tap water. Stain with haematoxylin for 3-4 minutes and wash in tap water. Allow the sections in tap water 5-10 min and wash in tap water. Counter stain with 0.5% until section appears light pink (15 to 30seconds), and then wash in tap water. Blot and dehydrate in alcohol. Clear with xylol (15 to 30 seconds). Mount in Canada balsam or DPX Moutant. Keep slide dry and remove air bubbles.

7. RESULTS AND DISCUSSION

7.1 RESULTS

7.1.1 PRELIMINARY PHYTOCHEMICAL SCREENIG

The results of the physiochemical, elemental analysis and quantitative estimation of phyto constituents followed by the pharmacological screening of various activities have been presented and discussed here below. In this study ethanolic extract of EPA (Extract of *plumeria acutifolia)* showed positive to following phytochemical constituent's alkaloids, carbohydrates, saponins, tannins, flavonoides, total phenolic substances, glycosides, carotenoids, fatty acids and terpenoids. The results are tabulated in Table 1.

Table. 8 Preliminary phytochemical screening of EPA (Extract of *Plumeria Acutifolia)*

Name of the Phytoconstituents	**…….extract**
Carbohydrates	+
Glycosides	+
Alkaloids	+
Phytosterol,steroids	-
Proteins and Amino acids	+
Flavanoids	+
saponins	+
Tri Terpenoids	+

(+) presence , (-) absence

7.1.2 PHARMCOLOGICAL STUDIES

Table.9 Body weight vs Mean Servival Time

Group	CONTROL	ONLY DAL	DAL+5HT	DAL+EXT 100mg/kg	DAL+EXT 200mg/kg
1ST WK	21± 1.34164	54± 33.6135	17.8333± 0.654047	57.6667± 32.8681	23.3333± 0.494413
2ND WK	24.3333± 0.918937	14.6667± 6.56083	24.6667± 0.954521	21.1667± 6.804	26.8333± 5.46758
3RD WK	26.6667± 0.666667	21.1667± 9.50935	27.8333± 1.6816	24.5± 7.86448	24.5± 7.77925

Values arae expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's(n=6); ns-non significant, $p>0.05$, **$p<0.01$, ***$p<0.001$, calculated by comparing treated groups with control group.

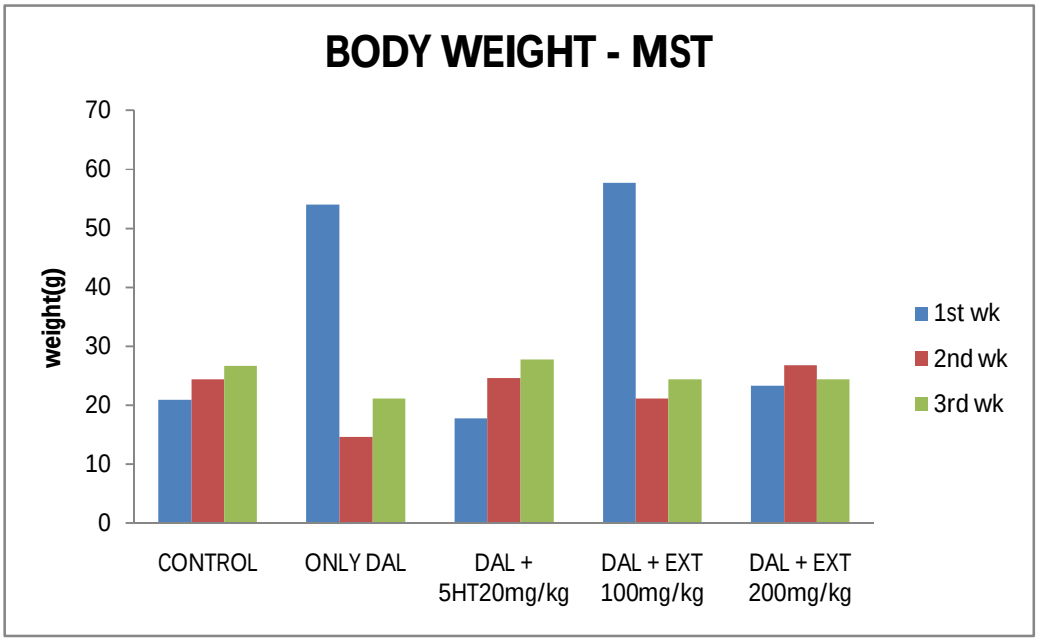


Figure.7 Effect of EPA on Body Weight vs MST

Table.10 Effect on MEAN SERVIVAL TIME

Group	CONTROL	ONLY DAL	DAL+5HT	DAL+EXT 100mg/kg	DAL+EXT 200mg/kg
MES	18.8333± 2.16667	12.8333± 1.16667	16.3333± 1.8738	15± 1.98326	18± 1.54919

Values arae expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's(n=6); ns-non significant, p>0.05, **p<0.01, ***p<0.001, calculated by comparing treated groups with control group.

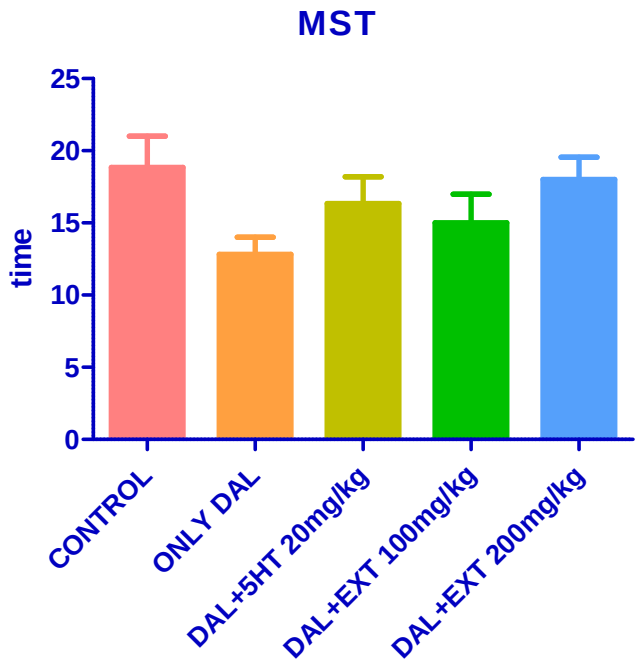


Figure.8 Mean Servival Time

Table.11 Effect on BODY WEIGHT

Group	CONTROL	ONLY DAL	DAL+5 FU 20mg/kg	DAL+Ext 100mg/kg	DAL+ Ext 200mg/kg
In body weight	19.3333± 1.11555	15± 0.447214***	16.3333± 0.614636*	22.3333± 0.333333*	21.6667± 0.614636^{ns}
Final body weight	24± 1.12546	25± 8.07878	16.6667± 5.33333	26.1667± 8.37622	19.1667± 6.14501

Statistical comparison: Each group (n=6), each value represents Mean ± SEM.One way ANOVA, followed by Dunnett comparison was performed. control group was compared with std group-II. ***(***P<0.001-**P<0.01, *P<0.05)*** treated groups III, IV,V was compared with group I.

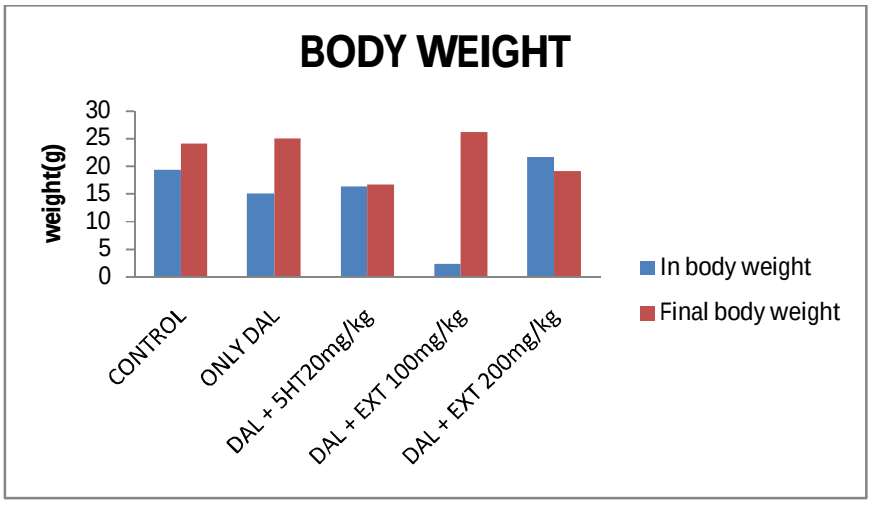


Figure.9 Body Weight

Table.12 Effect on TUMOR VOLUME

Group	CONTROL	ONLY DAL	DAL+5 FU 20mg/kg	DAL+Ext 100mg/kg	DAL+ Ext 200mg/kg
Tumor volume	0±0	7.86667± 2.8378	3.4± 2.05913	7.16667± 3.24722	4.08333± 1.96815

Statistical comparison: Each group (n=6), each value represents Mean ± SEM.One way ANOVA, followed by Dunnett comparison was performed. control group was compared with std group-II. ***(***P<0.001-**P<0.01, *P<0.05)*** treated groups III, IV,V was compared with group I.

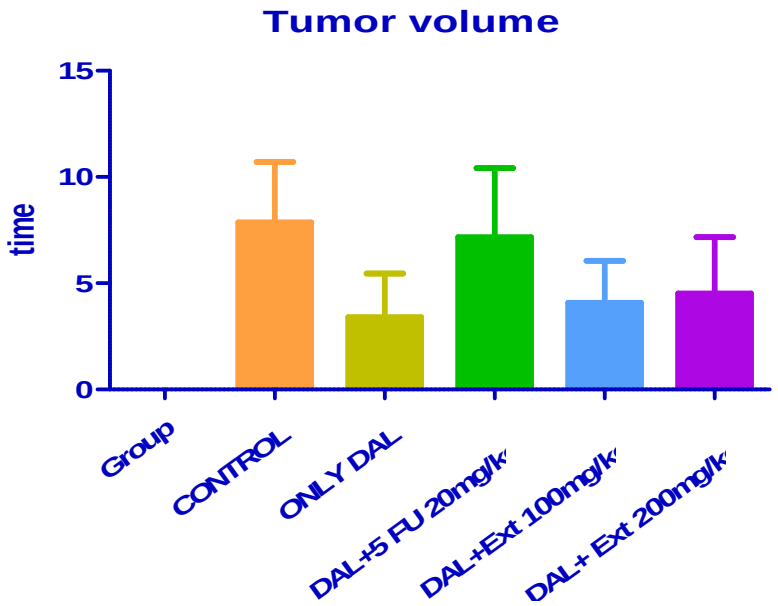


Figure.10 Tumor Volume

HEMATOLOGICAL PARAMETERS

Table.13 Effects on Hematological Parameter

Group	CONTROL	ONLY DAL	DAL+5 FU 20mg/kg	DAL+Ext 100mg/kg	DAL+ Ext 200mg/kg
Rbc	5.31±0.157987	4.93333±0.214051	4.45667±0.211702	4.95±0.291136	4.54±0.152883
Wbc	12.6±0.729383	35.5667±3.59153***	22.5±1.52883**	21.8667±1.53594**	31.6667±0.9895***
Total Haemoglobin	12.9333±0.470933	11.8±0.636658	10.3667±0.643256	11.8333±0.875087	10.5333±0.421637
Packed Cell Volume	39.8333±1.42797	36.5333±1.96141	32.2667±1.82805	36.1667±2.41449	32.5667±1.57917
Polymorphs	4.33333±0.760117	13±1.09545***	9±0.966092	7.66667±1.47573	13±1.93218***
Lymphocytes	89.3333±1.52023	81±1.31656***	83.6667±1.17379*	86.6667±1.83787	80.6667±1.83787***
Monocytes	2.66667±0.210819	4.33333±0.210819**	4±0.365148*	2.66667±0.421637	2.66667±0.210819
Eosinophils	3.66667±0.557773	5±0.365148	3.33333±0.557773	3±0.365148	3.66667±0.557773
Mch	26.1333±0.128236	25.3333±1.39778	21.8667±0.749518***	25.1667±0.310555	24.3667±0.243128

Statistical comparison: Each group (n=6), each value represents Mean ± SEM.One way ANOVA, followed by Dunnett comparison was performed. control group was compared with std group-II. ***(***P<0.001-**P<0.01, *P<0.05)*** treated groups III, IV,V was compared with group I.

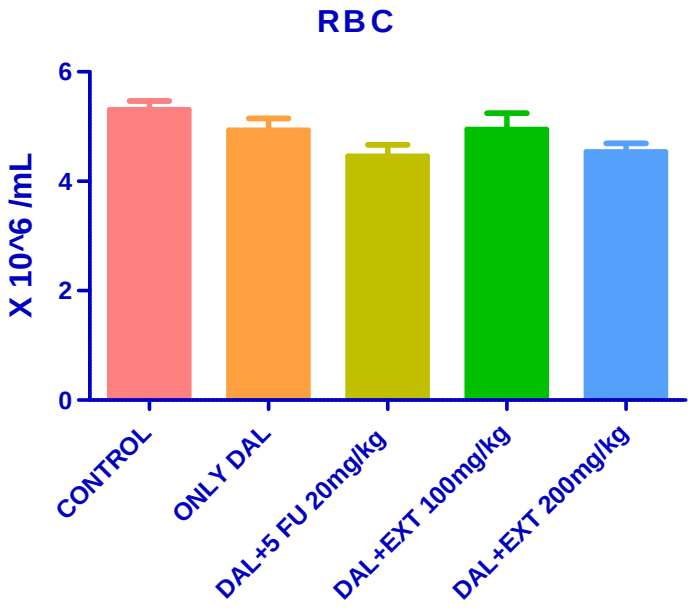


Figure.11 Effect on RBC

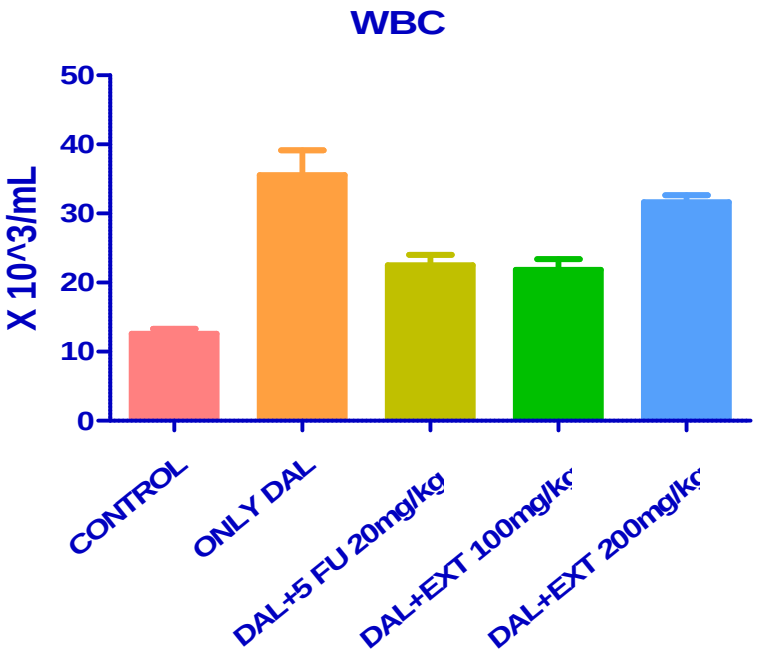


Figure.12 Effect on WBC

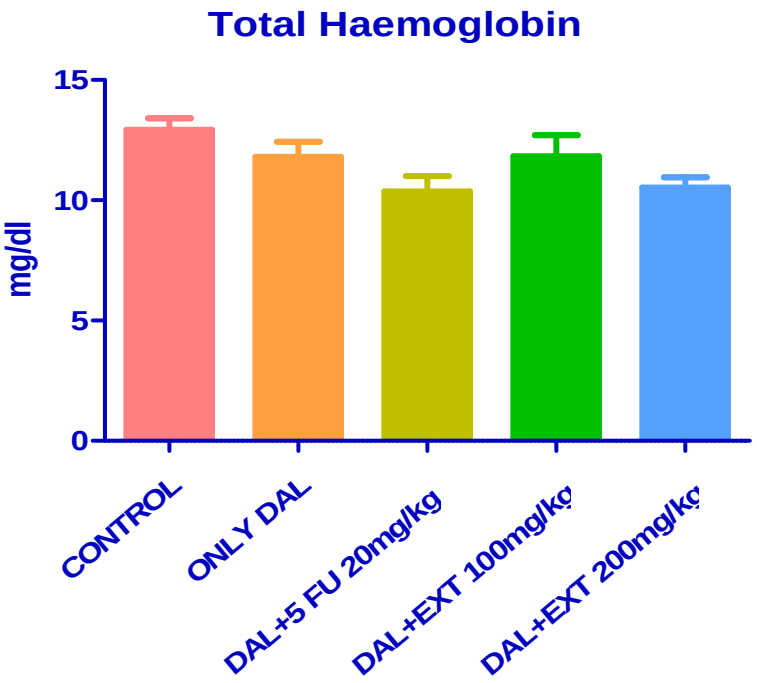


Figure.13 Effect on Total Hemoglobin

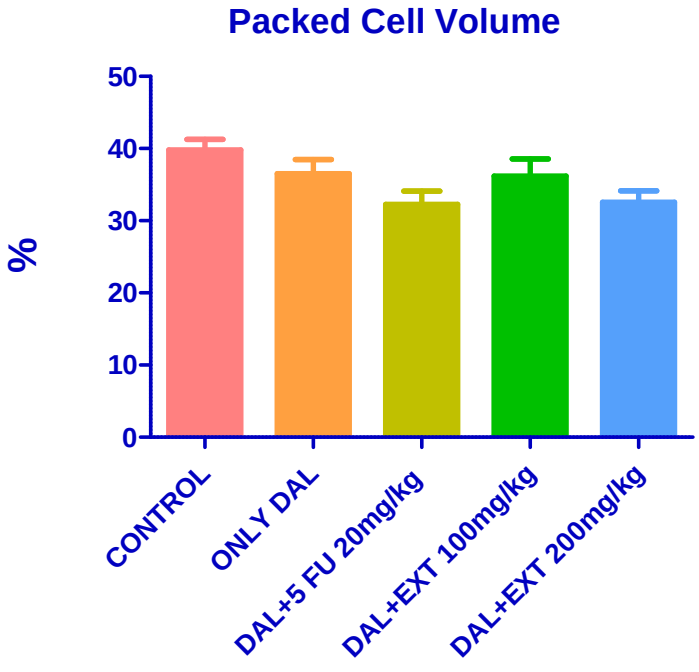


Figure.14 Effect on Packed Cell Volume

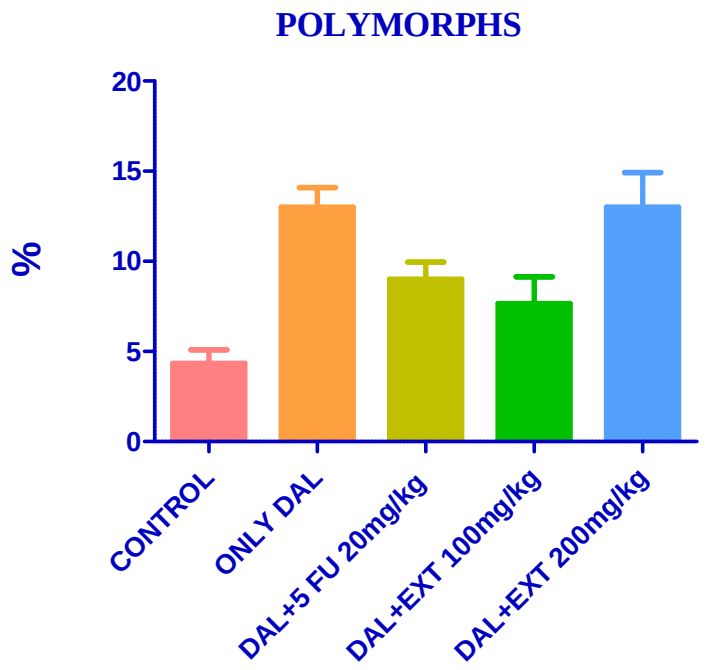


Figure.15. Effect on Polymers

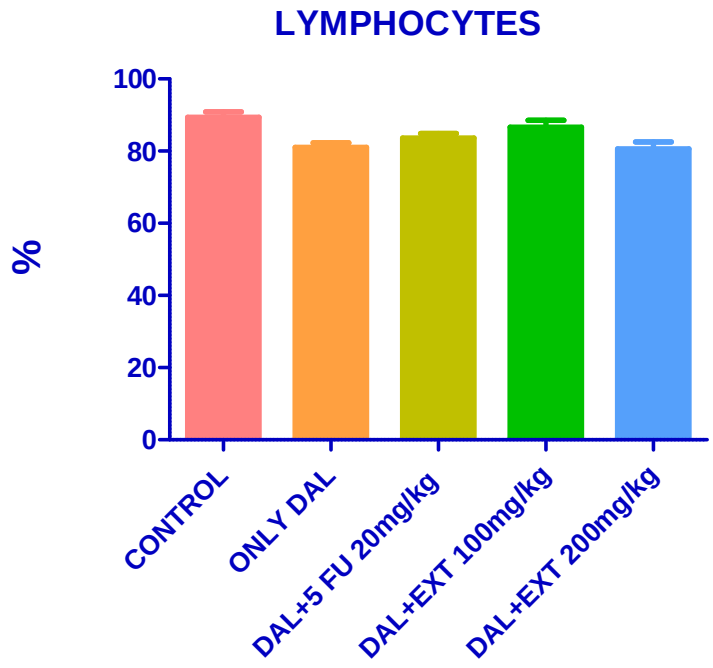


Figure.16 Effect on Lymphocytes

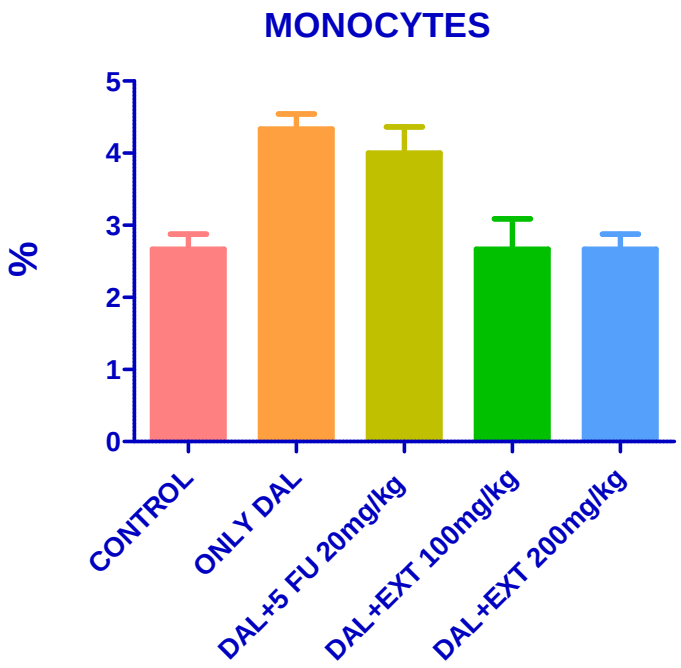


Figure.17 Effect on Monocytes

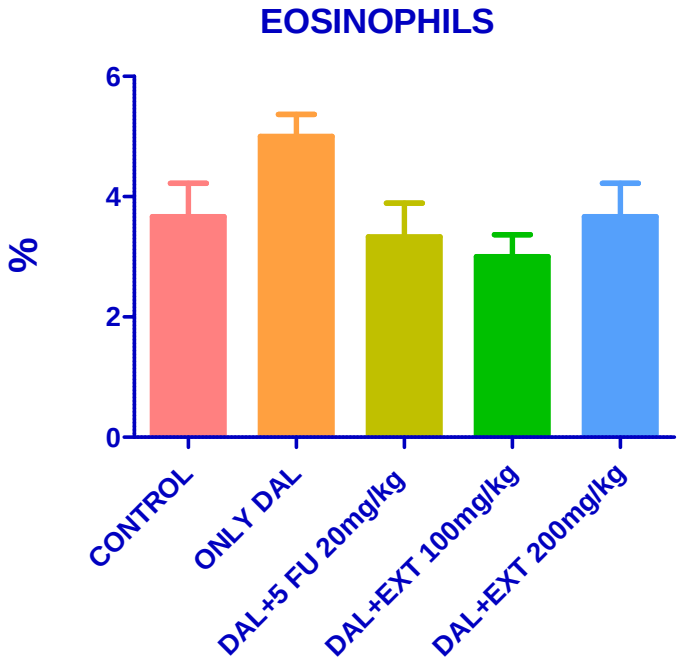


Figure.18 Effect on Eosinophils

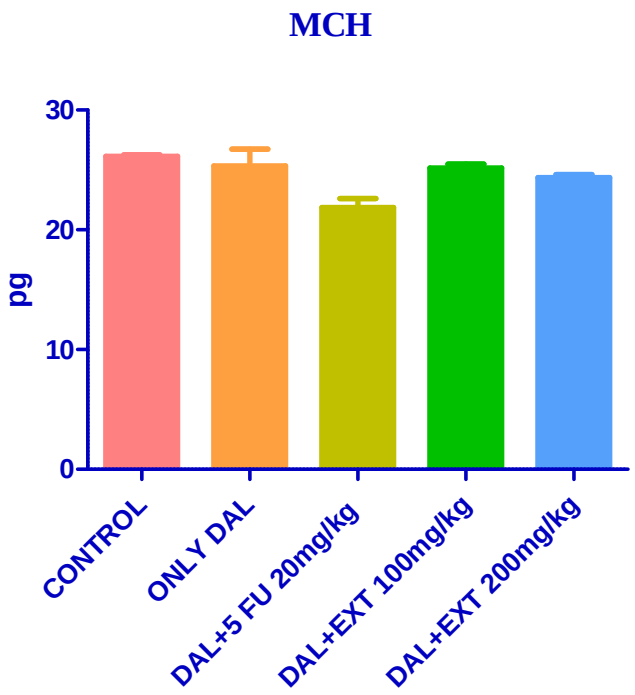


Figure.19 Effect on MCH

PACKED CELL VOLUME

Table.14 Effect On Packed Cell Volume

CONTROL	CONTROL	ONLY DAL	DAL+5HT 20mg/kg	DAL+EXT 100 mg/kg	DAL+EXT 200mg/kg
Packed Cell Volume	0±0	3.03333±0.641179***	0.923333±0.023758	3.1±0.334664***	2.7±0.109545***

Statistical comparison: Each group (n=6), each value represents Mean ± SEM.One way ANOVA, followed by Dunnett comparison was performed. control group was compared with std group-II. ***(***P<0.001-**P<0.01, *P<0.05)*** treated groups III, IV,V was compared with group I.

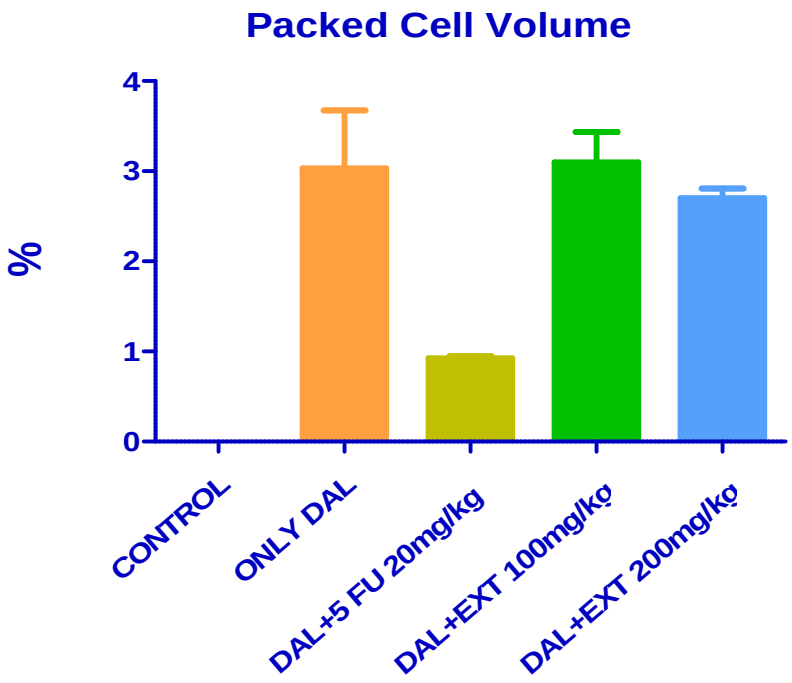


Figure.20 Effect on Packed Cell Volume

TOTAL PROTEIN CONTENT

Table.15 Effect on Total Protein Content

Group	CONTROL	ONLY DAL	DAL+5HT 20mg/kg	DAL+EXT 100 mg/kg	DAL+EXT 200mg/kg
Total protein (mg/dl)	6.73333±0.0760117	5.86667±0.0760117***	5.03333±0.0557773***	6.13333±0.18738**	6.2±0.0966092**

Statistical comparison: Each group (n=6), each value represents Mean ± SEM.One way ANOVA, followed by Dunnett comparison was performed. control group was compared with std group-II. ***(***P<0.001-**P<0.01, *P<0.05)*** treated groups III, IV,V was compared with group I.

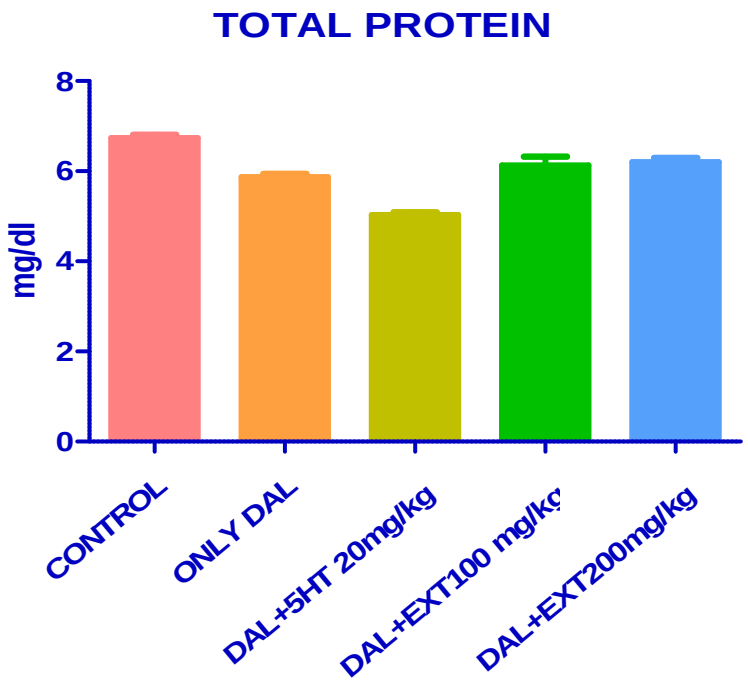


Figure.21 Effect on Total Protein

TOTAL BILRUBIN

Table.16 Effect on Total Bilrubin

Group	CONTROL	ONLY DAL	DAL+5HT 20mg/kg	DAL+EXT 100 mg/kg	DAL+EXT 200mg/kg
Total bilirubin (mg/dl)	0.616667±0 .0462361	0.953333±0. 0374759***	0.666667±0 .0402216	0.826667±0 .040387**	0.656667± 0.0314819

Statistical comparison: Each group (n=6), each value represents Mean ± SEM.One way ANOVA, followed by Dunnett comparison was performed. control group was compared with std group-II. ***(***P<0.001-**P<0.01, *P<0.05)*** treated groups III, IV,V was compared with group I.

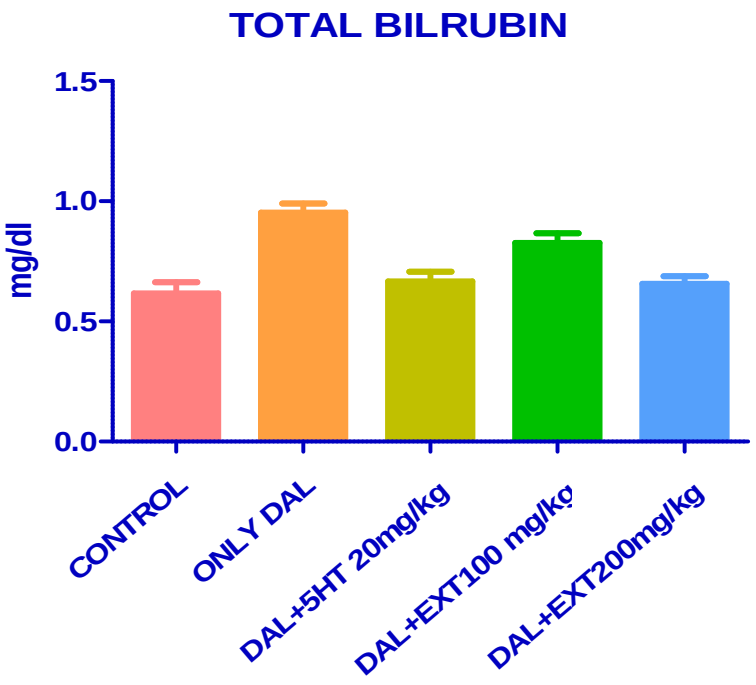


Figure.22 Effect on Total Bilrubin

LIVER ENZYMES

Table.17 Effect on Liver Enzymes

Group	CONTROL	ONLY DAL	DAL+5HT 20mg/kg	DAL+EXT100 mg/kg	DAL+EXT200mg/kg
SGOT(U/L)	87.7± 2.35542	205.367± 23.0248***	173.4± 10.8622***	199.4± 8.96423***	170.467± 5.72088***
SGPT(U/L)	61.1± 2.41744	59.8333± 3.36736	77.8± 9.12403	71.1333± 8.55619	55.6± 7.7439
ALP (U/L)	146.233± 8.01352	19.3333± 2.29211	27.8± 2.94211	58.9367± 16.1998	38.4± 6.27981

Statistical comparison: Each group (n=6), each value represents Mean ± SEM.One way ANOVA, followed by Dunnett comparison was performed.

control group was compared with std group-II. *(***P<0.001-**P<0.01, *P<0.05)* treated groups III, IV,V was compared with group I.

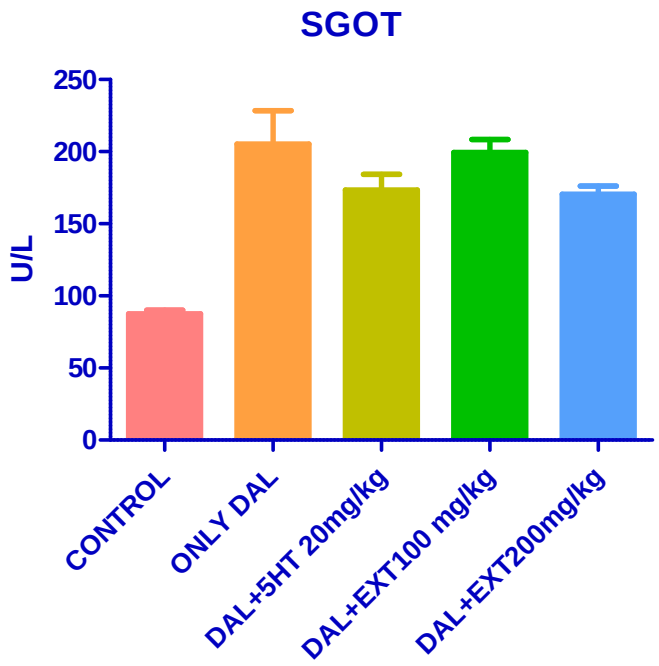


Figure.23 Effect on Serum Glutamate Oxalo Acetate Transaminase

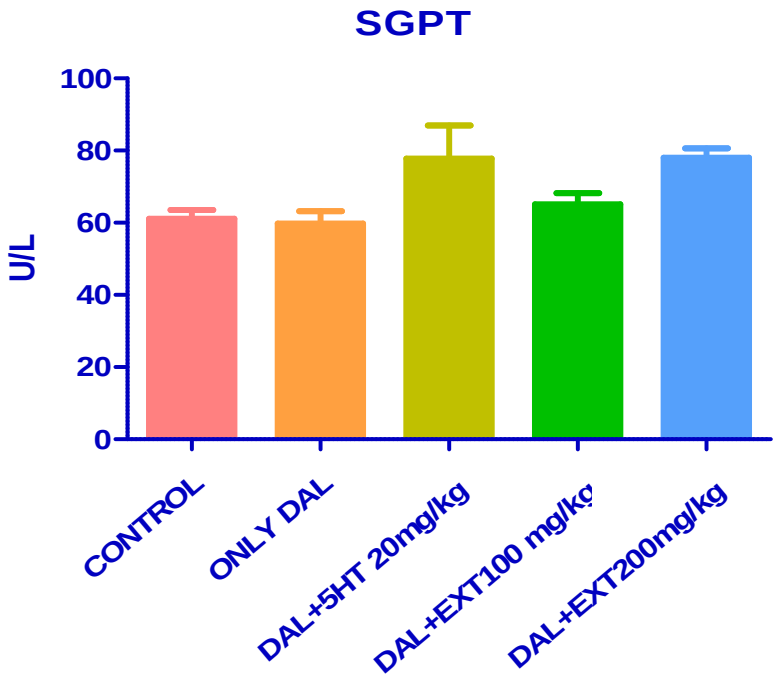


Figure.24 Effect on Serum Glutamate Pyruvate Transaminase

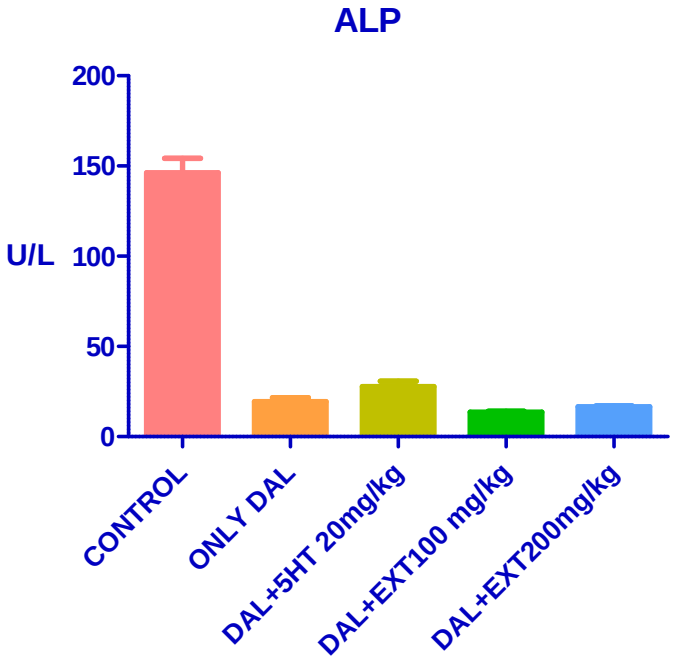


Figure.25 Effect on Alkaline Phosphatase

URINARY CONTENTS

Table.18 Effect on Urinary Content

Group	CONTROL	ONLY DAL	DAL+5HT 20mg/kg	DAL+EXT100 mg/kg	DAL+EXT200 mg/kg
UREA (mg/dl)	37.6667± 2.85315	55.4667± 3.00762	57.4333± 3.80006	81.1± 5.52606***	40.1± 0.438
CREATINE (mg/dl)	0.9033± 0.08826	0.3033± 0.0117***	0.5033± 0.038	1.136± 0.0947***	0.37± 0.02***
Uric acid (mg/dl)	2.203±0.137421	1.157±0.118678***	2.233±0.08558	1.183±0.08052***	2.383±0.171

Statistical comparison: Each group (n=6), each value represents Mean ± SEM.One way ANOVA, followed by Dunnett comparison was performed. control group was compared with std group-II. ***(***P<0.001-**P<0.01, *P<0.05)*** treated groups III, IV,V was compared with group I.

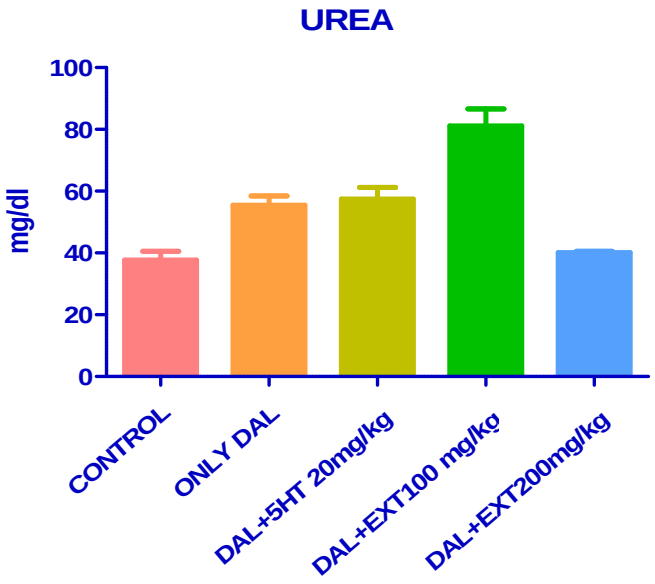


Figure.26 Effect on Urea

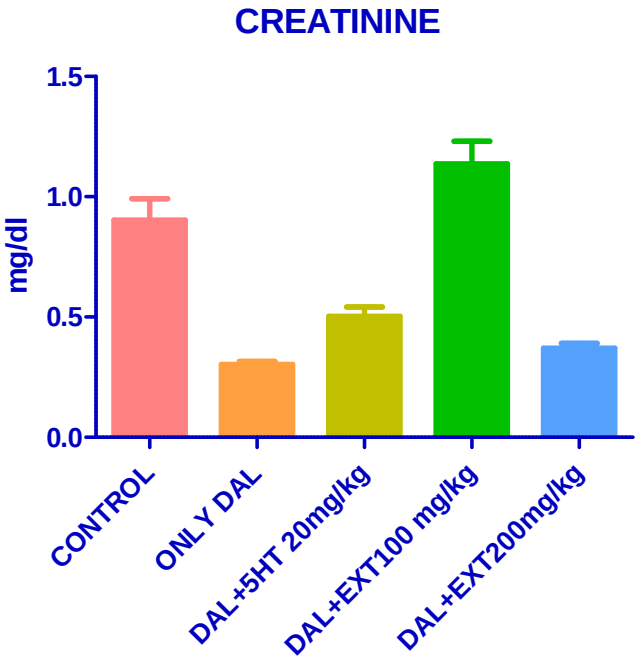


Figure.27 Effect on Creatinine

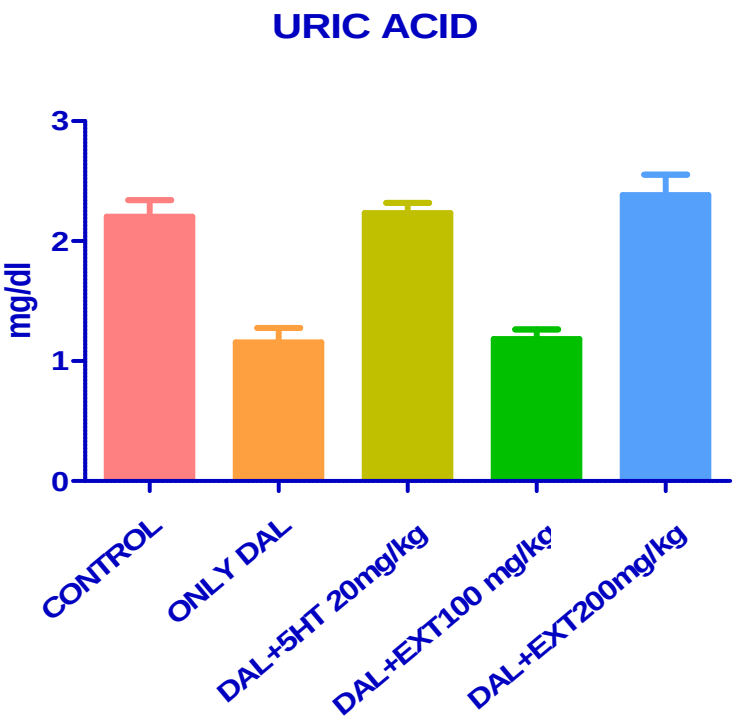


Figure.28 Effect on Uric Acid

LIPID PROFILE

Table.19 Effect on Lipid Profile

Group	CONTROL	ONLY DAL	DAL+5HT 20mg/kg	DAL+EXT100 mg/kg	DAL+EXT200mg/kg
Total cholesterol (mg/dl)	76.3±2.80785	92.5333 ± 6.98669	98.6± 12.1218	63.1± 7.18563	90.1333± 9.12757
triglycerides (mg/dl)	60.9±5.27295	90.5333 ± 12.9007	175.2± 32.6986**	25.8± 6.45972	142.133± 38.7123*
HDL-Cholesterol (mg/dl)	46.6667± 1.73449	43.7667 ± 1.46075	34.6333± 4.3418**	35.8333± 1.92435*	39.6667± 0.713987

Statistical comparison: Each group (n=6), each value represents Mean ± SEM.One way ANOVA, followed by Dunnett comparison was performed. control group was compared with std group-II. ***(***P<0.001-**P<0.01, *P<0.05)*** treated groups III, IV,V was compared with group I.

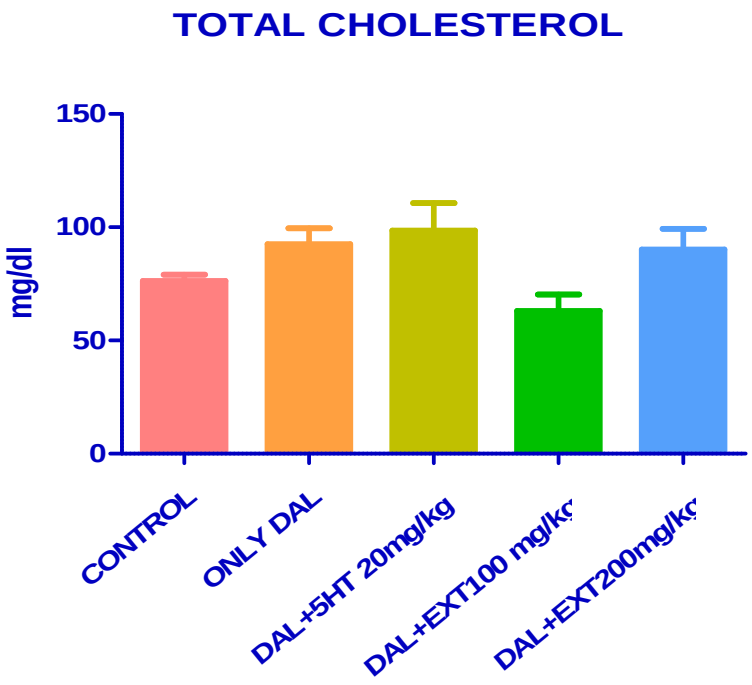


Figure.29 Effect on Total Cholesterol

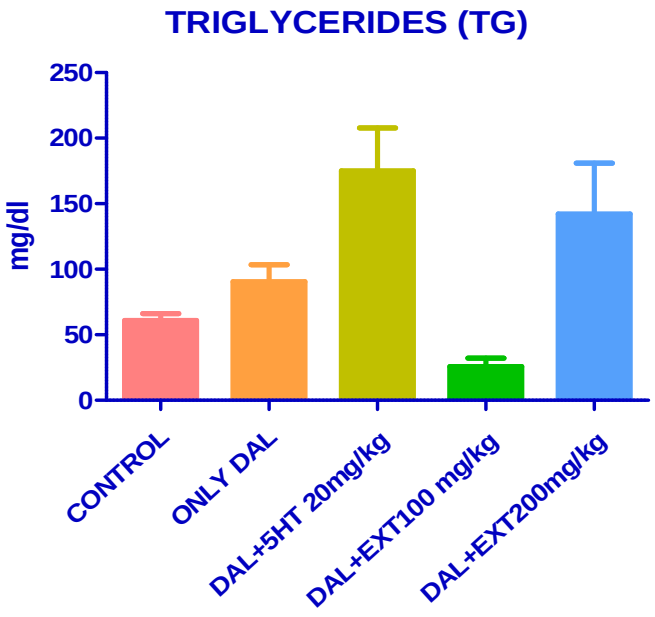


Figure.30 Effect on Triglycerides

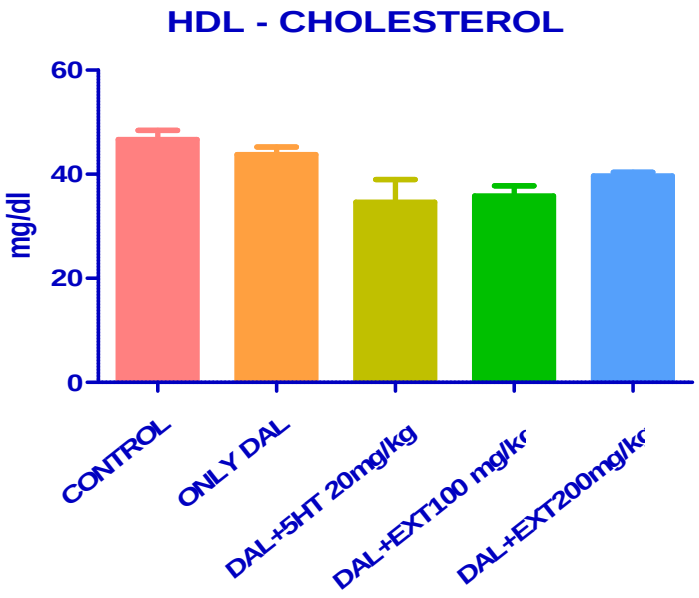


Figure.31 Effect on High Density Lipoprotein

EFFECT OF EXTRACT ON ANTIOXIDANT LEVELS

Table.20 Effect on anti Oxidant Level

Group	CONTROL	ONLY DAL	DAL+5HT 20mg/kg	DAL+EXT 100 mg/kg	DAL+EXT200mg/kg
SOD	5.71±0.50	2.70±0.30	6.23±0.25	4.73±0.17	3.96±0.18
Catalase	86.17±3.92***	50.17±2.31***	87.17±2.85***	74.50±1.87***	65.00±2.36***
LPO	8.85±0.81***	36.50±1.51***	13.50±1.87***	24.67±2.50***	20.17±0.98***
GPx	45.50±2.42***	20.67±1.21***	50.17±2.13***	35.00±2.00***	41.67±1.96***
GSH	32.67±1.63***	14.67±1.03***	36.33±1.63***	19.67±1.03***	30.17±0.98***
Vitamin C	1.30±0.10***	0.27±0.04***	1.21±0.10***	0.75±0.05***	0.96±0.16***

Values are expressed as mean ± S.D. Statistical significance (p) was calculated by one way ANOVA followed by Tukey multiple comparisontest. Control group was compared with normal. Standard and test groups were compared with control. $^{***}P<$ 0.05wasconsideredsignificant.

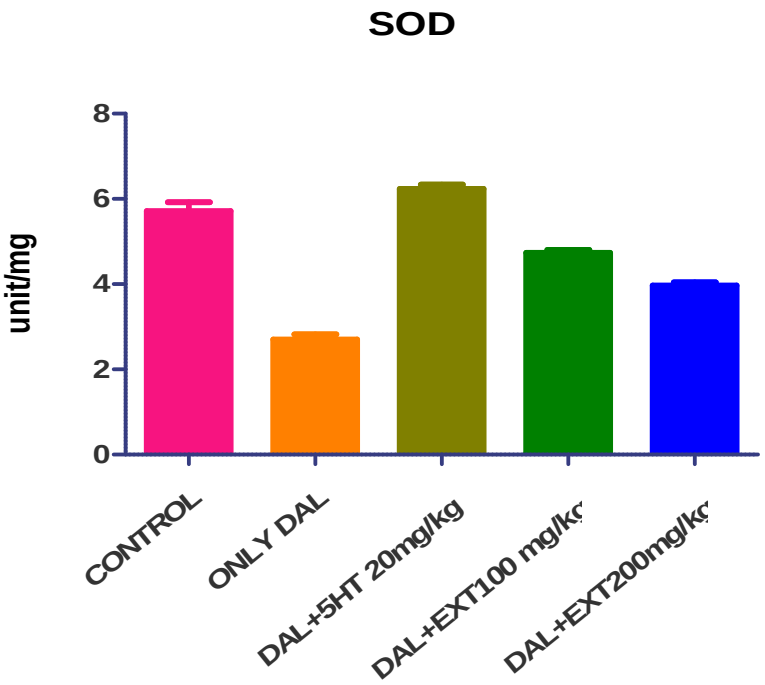


Figure.32 Effect on Superoxide Dimutase

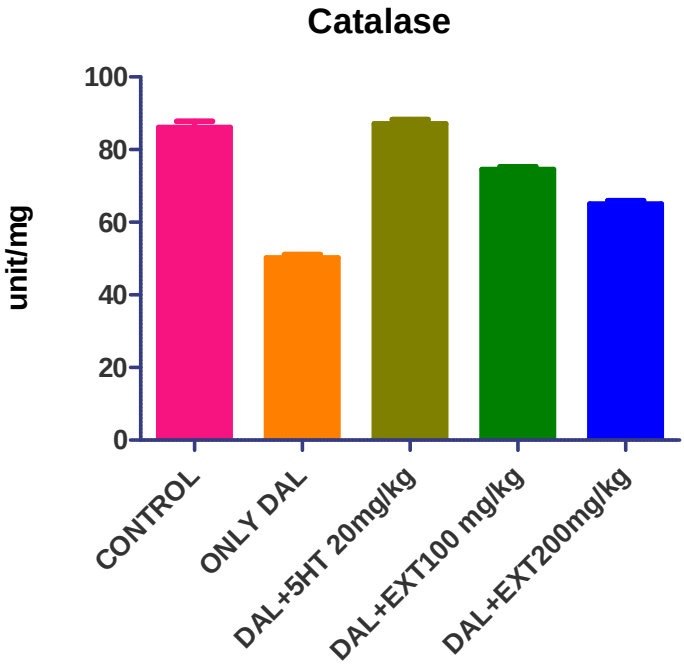


Figure.33 Effect on Catalase

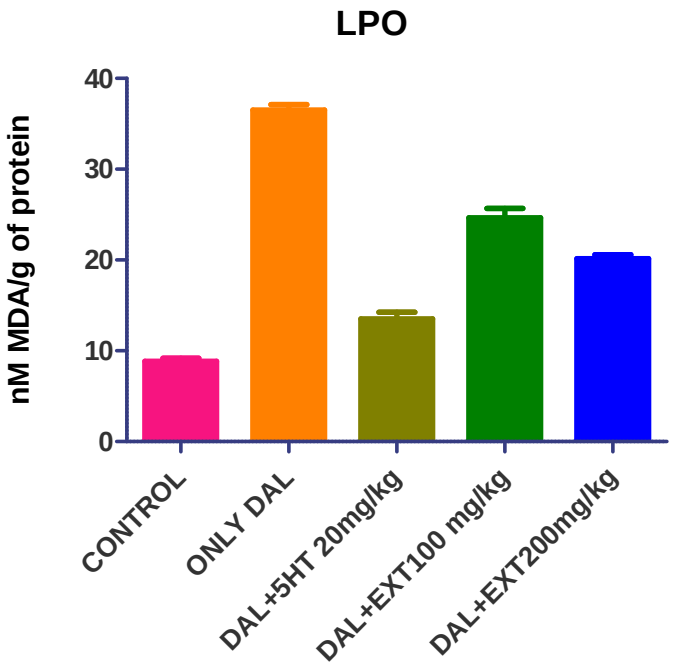


Figure.34 Effect on Lipid Peroxidation

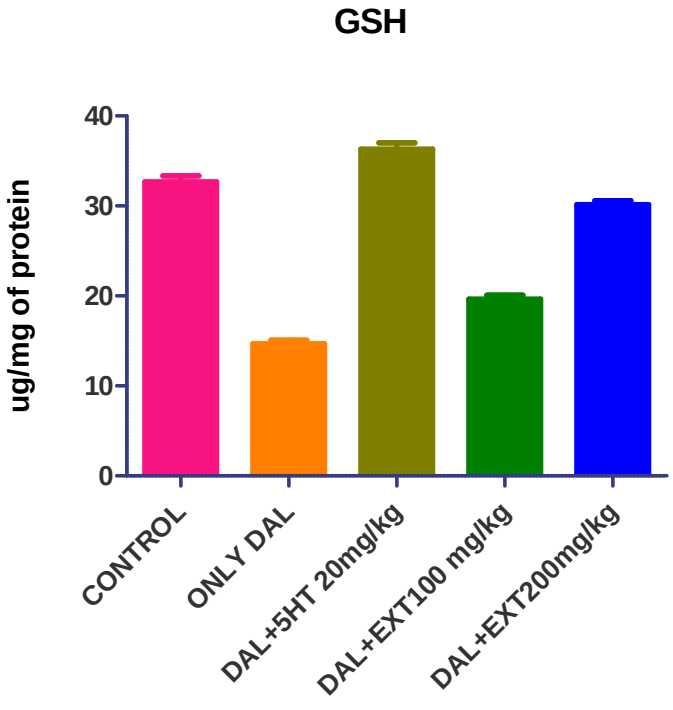


Figure.35 Effect on Glutathione

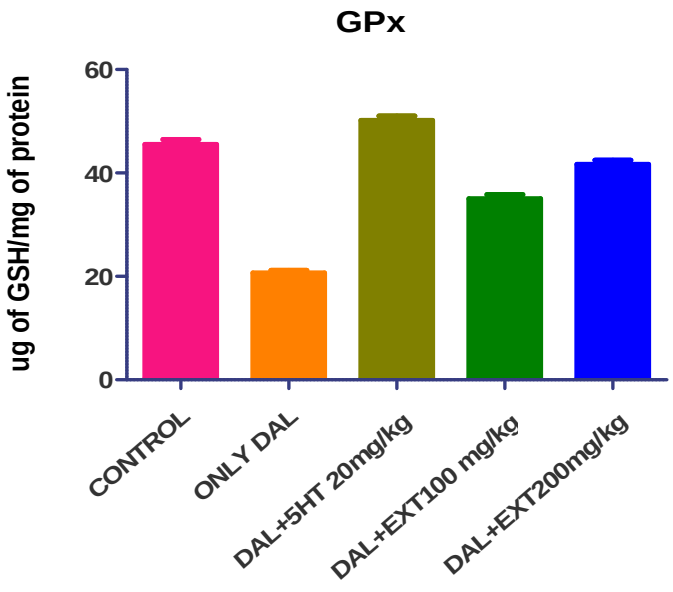


Figure.36 Effect on Glutathione Peroxidase

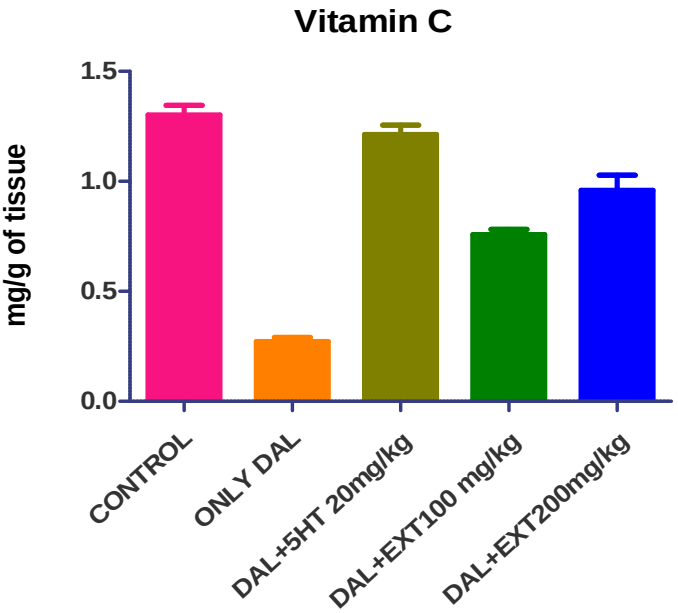


Figure.37 Effect on Vitamin C

7.1.3 HISTOPATHOLOGY

GROUP - I CONTROL

Section studied from the liver shows normal lobular architecture. Hepatocyte shows normal morphology with foci of lobularinflammation.Contral vein shows normal.

GROUP – II ONLY DAL

Section studied from the liver shows preserved lobular architecture.Few foci shows lympoplasmacytic infiltration with proliferation of thin walled vessels.Hepatocyte shows normal morphology. Portal tract shows normal.Contral vein shows mild dilatation.Sinusoids show dilatation.

GROUP – III DAL+ 5FU 20mg/kg

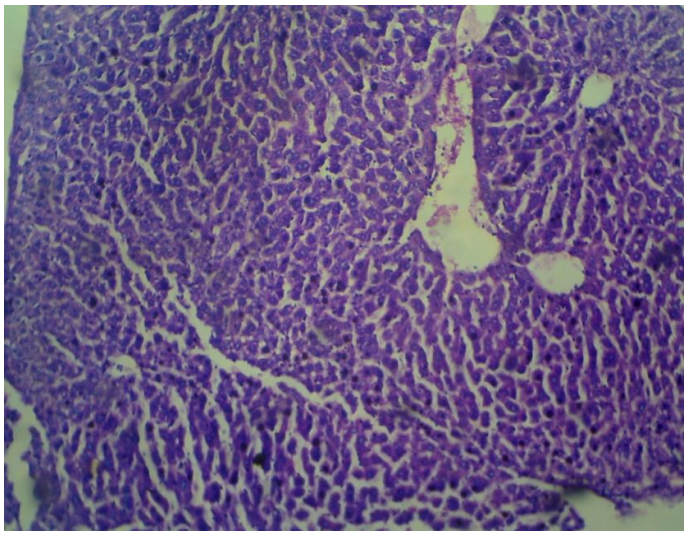
Section studied from the liver shows maintained lobular architecture. Hepatocyte shows normal morphology. Portal tract shows normal.Contral vein and Sinusoids shows mild dilatation.There is no evidence of inflammation/necrosis.

GROUP - IV DAL+ EXT 100mg/kg

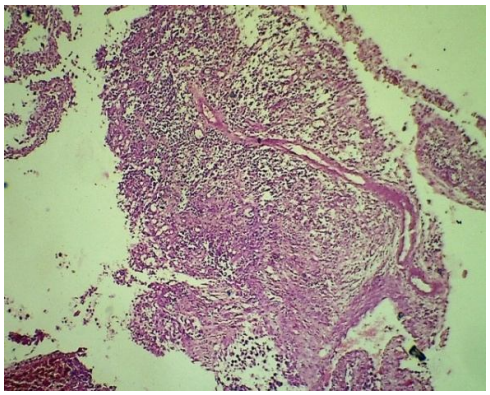
Section studied from the liver shows maintained lobular architecture. Hepatocyte shows normal morphology with foci of lobularinflammation.Portal tract shows mild lymphocytic infiltration. Contral vein shows normal.Sinusoids show mild dilatation.

GROUP – V DAL + EXT 200mg/kg

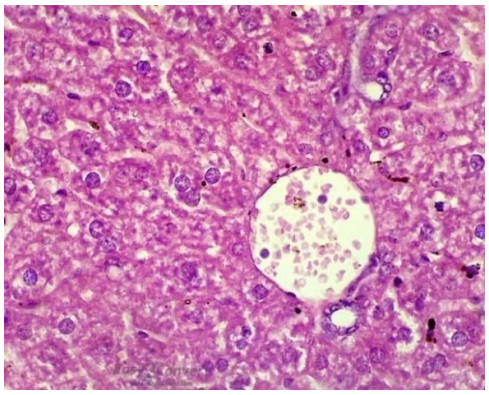
Section studied from the liver shows maintained lobular architecture. Hepatocyte shows normal morphology. Portal tract shows normal. Contral vein shows normal. Sinusoids show mild dilatation.Peripheri shows dense lymphoplasmacytice infiltration is seen.



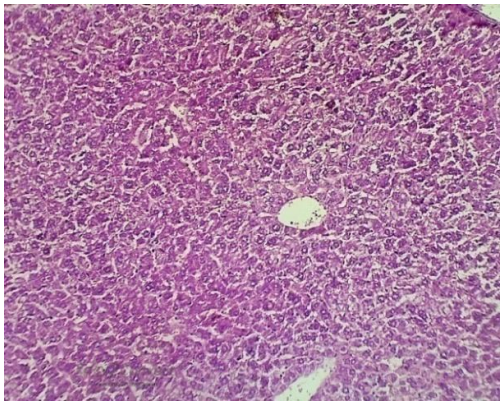
CONTROL



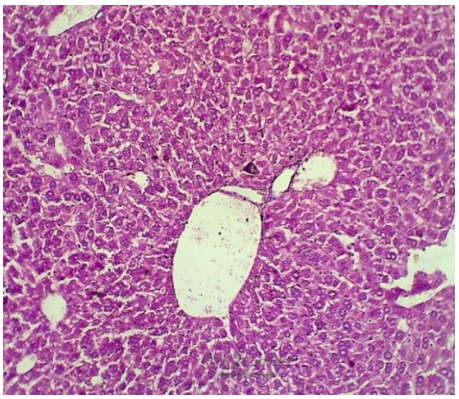
ONLY DAL



DAL+ 5FU20mg/kg



DAL+ EXT100mg/kg



DAL + EXT200mg/kg

Figure.38 Histopathology of DAL induced and EPA treated cells

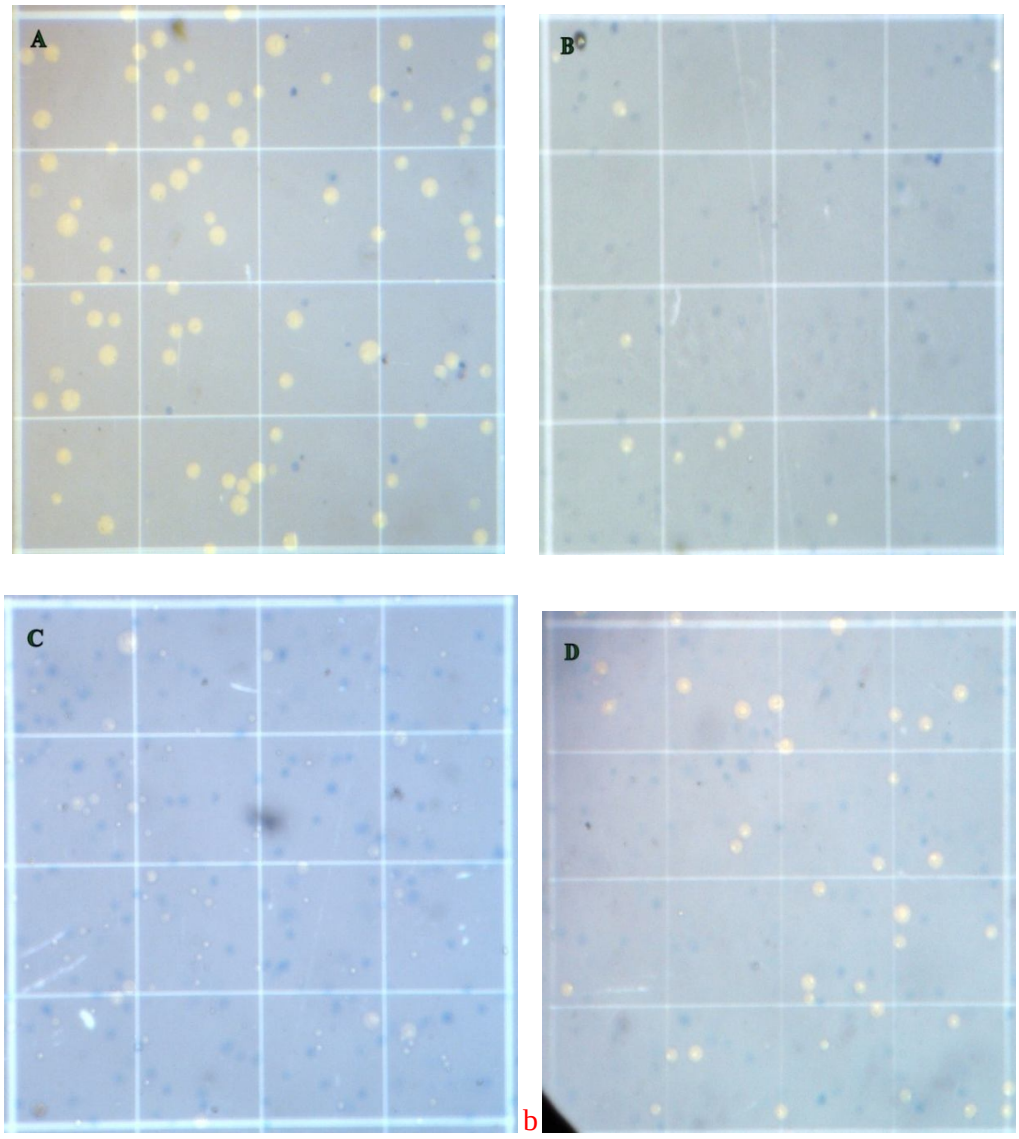
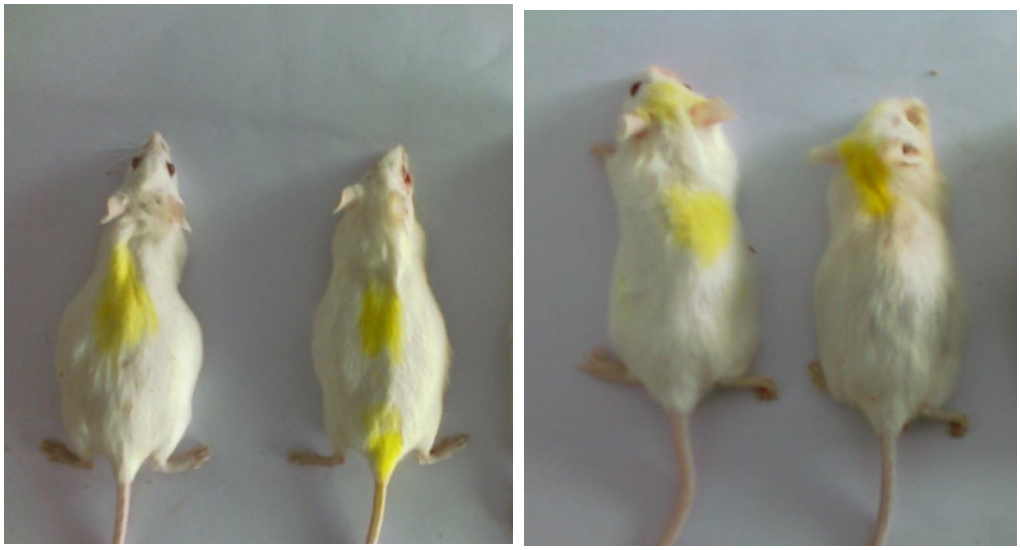


Figure.39 Shows Effect Of Map On Viable And Non-Viable Cell Count after 13 days treatment A: DAL control; B: DAL + 5-FU, 20 mg/kg body weight; C: DAL +EPA, 100 mg/kg body weight; D: DAL + MAP,200 mg/kg body weight

ONLY DAL DAL+ 5FU 20mg/kg ip



DAL + EPA 250mg/kg (p.o) DAL + EPA 500mg/kg(p.o)

Figure.40 EFFECT OFETHANOLIC EXTRACT OF *Plumeria acutifolia* Poir ON DAL INDUCED CANCER IN MICE

7.2 DISCUSSION

The **preliminary phytochemical studies** of EPA indicated the presence of several triterpenoids, phenols, Flavonoids, tannins, glycosides, and so on. The observed antitumor, hepatoprotective, and antioxidant activities may be due to the presence of any of these compounds in EPA.

Bioactive antioxidant levels:

Flavonoids are a group of effective antioxidants which are present abundantly throughout the plant kingdom. Flavonoid and related compound are effective in scavenging DPPH radical, hydroxyl radical and in metal-chelating capacity Flavonoids are found to exhibit numerous biological activities like vasodilator, anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, antiallergic, and antiviral effects [14, 15].

Tannins, the high molecular weight phenols, act as a good scavenger of free radical either by donating hydrogen atom or by reducing them. This property is attributed by the molecular weight, the number of aromatic rings and nature of hydroxyl group's substitution and specific functional groups present in the tannins. The results show that the methanol extract contains more tannin.[55]

Phenols are very important plant constituents because of their radical scavenging ability due to the hydroxyl groups. **Polyphenols** are known to exhibit a variety of biological actions such as free radicals scavenging, metal chelation, modulation of enzyme activity and more recently to effect signal transduction, activation of transcription factors and gene expression.[56]

DAL-induced ascitic antitumor studies

The reliable criteria for judging the value of an anticancer drug is the prolongation of the life span of animals. In DAL-tumor-bearing mice, a regular rapid increase in ascitic tumor volume was observed[62]. The DAL-bearing mice orally administered EPA at 100 and 200 mg/kg body weight showed significant change in the average life span compared to animals of the tumor control group. However, the percent increase in body weight, tumor cell volume, and number of viable tumor cells were found to be significantly less than the tumor control animals, indicating the

anticancer nature of the extract enumerated in the **table no.12**. These results could indicate either a direct cytotoxic effect of EPA on tumor cells as evidenced by the in vitro studies or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition. Hence, the observed antitumor nature of EPA may be due to the cytotoxic properties.

In cancer chemotherapy the major problems are of myelosuppression and anaemia[70,71]

Anaemia encountered in tumour bearing mice is mainly due to reduction in RBC and HB% and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions.[72] However, the elevation of WBC levels may be due to its adverse effect on the haemopoietic system[58]

The reversal of Hgb content, RBC, Total WBC, and differential count of WBC by the EPA treatment towards the values of the normal group clearly indicates that EPA possessed protective action on the haemopoietic system (**Table.13**).

Biochemical parameters: The inoculation of DAL cells caused significant increases in the levels of TGL, TP, TC, ALP[61] and ALBUMIN and there is no significant change in the levels of ASAT and ALAT in the serum of tumor control animals, when compared to the normal group.The treatment with EPA at 100 and 200 mg/kg body weight reversed these changes towards the normal levels (**Table.14 to 17**). Most of the values were found to be significant. The treatment with standard 5-FU also gave similar results.

The antioxidant nature of EPA was also evident by the in vitro studies. Plants with high total phenol content are known to possess strong antioxidant properties[60]. The observed antioxidant activity may be due to the high phenolic content of the extract.

It was observed that tumor cells produced more peroxides when they proliferate actively after inoculation of tumor. This rise in peroxides indicated the occurrence of intensification of oxygen free radical production[62]. Cells which are equipped with enzymatic antioxidant mechanisms play an important role in the elimination of free radicals. High levels (up to 0.05 µmol/h per 104 cells) of H2O2 are constitutively released from a wide variety of human tumors [64].

Lipid peroxidation mediated by free radicals considered being a primary mechanism of cell membrane destruction and cell damage. The oxidation of unsaturated fatty acids in biological membrane leads in reduction membrane fluidity and disruption of membrane structure and function. MDA, the end product of lipid peroxidation was also reported to be higher in carcinomatous tissue than in non-diseased organs. Increase in levels of TBARS indicate enhanced lipid peroxidation leading to tissue injury and failure of antioxidant defense mechanism to prevent the formation of excess free radicals.

Administration of EPA significantly decreases the liver TBARS. **Table.20 represents LPX levels**.

The active role of GSH against cellular lipid peroxidation has been well recognized and there by reduces the glutathione (GSH) activity.GSH can act either to detoxify activate oxygen species such as H_2O_2 or reduce lipid peroxidation and increased the levels of glutathione content and there by it may act as an anti tumor agent.[63]

Administration of EPA significantly decreases the liver glutathione. **Table.20 represents GSH levels**.

On the other hand, SOD is aubiquitous chain breaking anti oxidant and is found in all aerobic organisms. It is mettalo protein widely distributed in all cells and plays an important protective role against ROS-induced Oxidative damage. The radical scavenging system catalase, which are present in all major organsims in body of animals and human beings and is especially concentrated in liver and erythrocytes . Both enzymes play an imp role in the elimination of ROS derived from the redox process of xenobiotic in liver tissues. It was suggested that catalase and SOD are easily inactivated by lipid peroxidase or ROS in correlation it has been reported that DAL bearing mice shows decreased levels of SOD activity and this may be due to loss of MN^{++} SOD activity in, liver inhibition of catalase activity in tumor cell lines also reported[65,66]. In this study, catalase and SOD were appreciable elevated by administration of EPA **(Table.20 represents SOD and Catalase levels)**.

Histopathological analysis

Histological observation of liver under a light microscope was done to observe the effect of MAP on the structural integrity of the cells.

The liver of normal animal showed normal histological appearance (Figure.38).

The tumor control animal liver showed slight enlargement of hepatocytes, dilated sinusoidal spaces containing and portal triads showing collections of lymphocytes

The animals treated with standard 5-Fu at 20 mg/kg i.p shows normal histological appearance of liver cells with no lymphocytes in the portal area

The animals treated with EPA at 100mg/kg and 200mg/kg body weight showed almost normal histological appearance of liver cells, except for a few lymphocytic collections in the portal area

8.SUMMARY AND CONCLUSION

The present dissertation work was carried out to evaluation of anticancer activity of ethanol extract of *plumeria acutifolia* leaf against Dalton's ascites lymphoma (DAL) induced cancer in mice. plumeria acutifolia were extracted with ethanol and the chemical constituents of the extract were identified by qualitative analysis.Preliminary phytochemical analysis of *plumeria acutifolia* showed the positive test of as carbohydrate, alkaloid, glycosides, saponins, flavanoids, triterpenoids, proteins.

Further anticancer and antioxidant studies of EPA against dalton's ascites lymphoma (DAL) in cancer mice increased the life span by arresting the tumor growth. Administration of EPA of the dose of 100 and 200 mg / kg in DAL bearing mice significantly increased the haemoglobin level,RBC count as well as reduce in WBC count. when comparision with DAL induced and after administration of the 100 and 200 mg of EPA extract shows decrease in LPX, decreases the GSH level and elevated SOD level.

These all results shows that the drug *plumeria acutifolia* confirmed to have anticancer activity and antioxidant activity may due to the presence of saponins, flavanoids, triterpenoids, etc.

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